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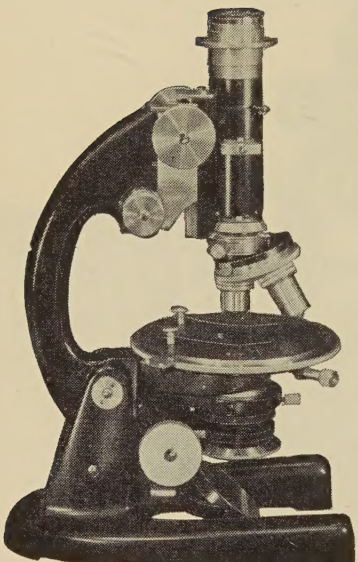

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Some Histochemical Observations on Amino-acids and Nucleic Acids in the Wool Follicle

By M. L. RYDER

(*From the Wool Industries Research Association, Torridon, Headingley, Leeds* 6)

With one plate (fig. 1)

Summary

A study of staining at different pH values showed that the fibre became decreasingly basiphil at successively higher levels. This was supported by an increased reaction for basic groups after keratinization, although a reaction for acid groups was obtained in the normally basiphil pre-keratinization region, and the keratinized fibre after vigorous treatment.

The reactions for individual amino-acid residues are probably not quantitative, but indicate availability to react. The strongest reaction for arginine was in the trichohyalin of the medulla and inner sheath, for tyrosine and tryptophan in the keratinized part of the fibre, and for histidine in both the keratinized and unkeratinized parts.

Most reactions gave asymmetrical results, but vigorous treatment tended to produce uniform results. This supports the suggestion that the asymmetry is due to differences in accessibility rather than to chemical differences.

Introduction

WITH 'sacpic' stain (Auber, 1952), the wool fibre, at successively higher levels, is first acidophil, staining green with the mixture of yellow picric acid and blue indigo-carmine. Then there is a short (pre-keratinization) region, which stains red with basic fuchsin or safranin, and finally the fully keratinized fibre stains yellow with picric acid. From these observations Auber suggested that there was first an excess of basic groups and then an excess of acidic groups, in the proteins of the unkeratinized fibre. Odland (1953) studied the ability of the human hair follicle to bind acid and basic dyes (orange G and methylene blue respectively).

So far as I know there are reliable histochemical tests for only 4 of nearly 20 amino-acids in the wool fibre. These are tyrosine, tryptophan, histidine, and arginine; it is their reactive side chains and the guanidine group of arginine that make detection possible. Tests for other amino-acids involving the amino- or carboxyl-groups are not in general suitable for the recognition of amino-acids forming part of a protein molecule.

Material and Methods

Dye-binding capacity

The method of Odland was used. Indigo-carmine and basic fuchsin were tried in addition to the stains used by Odland. Only wax sections of sheep-skin were used in this part of the study, but for all subsequent tests, unless

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otherwise stated, fresh-frozen and frozen-dried sections were used in addition to material fixed in 5% formalin. Where two or more methods for the same chemical group were available, the results from each were compared by using alternate sections.

Acidic and basic groups

The Gram reaction was used for acid (carboxyl) groups, which are indicated by a Gram-positive stain (Pearse, 1953). Basic (amino) groups were detected by four methods. As formalin reacts with these, either fresh or Zenker-fixed material was used.

First, slides were left overnight in the following reagent: ninhydrin 20 g, hydrindantin 3 g, methyl cellosolve 750 ml, acetate buffer (pH 5) 250 ml. The use of Schiff's reagent showed that sections treated with ninhydrin produced no greater aldehyde reaction than untreated slides (contrast Burstone, 1955). Secondly, slides were treated overnight at about 4° C, in a saturated (about 2%) solution of 1-fluoro-2:4-dinitrobenzene (FDNB) in 90% alcohol saturated with sodium bicarbonate. Thiol groups were blocked beforehand with mercuric chloride, and any non-specific yellow staining due to the decomposition product, dinitrophenol, was removed with dilute HCl. Thirdly, 3-hydroxy-2-naphthaldehyde prepared by the method of Weiss, Tsou, and Seligman (1954) was used in Hopman's (1957) procedure. This reagent alone produced no reaction; a colour was obtained by coupling with fast blue. Fourthly, the only reaction obtained with β-naphthaquinone sulphonate was a general brown coloration, apparently due to non-specific staining.

Amino-acid residues

All methods used were from Pearse (1953) unless otherwise stated. The Sakaguchi reaction for *arginine* seems to be the only one for this amino-acid whereas there are several for *tyrosine*. Those used were Millon's reagent for 16 to 24 h at room temperature, nitrosation followed by coupling (Lillie, 1957), and azo-dye coupling (described below). The use of FDNB followed by reduction, diazotization, and coupling was not developed because thiol and amino-groups have to be blocked before the reaction; when quinone was used to block both groups in one operation the sections were stained a dark colour that would have masked any further staining. The indole reaction gave only a pale coloration for *tryptophan*, but when the *p*-dimethylaminobenzaldehyde (DAB) was followed by sodium nitrite (Adams, 1957), a moderate blue colour developed. The method of Bruemmer and others (1957) seems to be a nitrosation rather than a diazotization and is thus similar to the nitrosation method for tyrosine. The coupling compound used in the tryptophan method is N-(1-naphthyl) ethylenediamine dihydrochloride (NED). The authors recommend Zenker fixation, but we obtained only a weak reaction with Zenker-fixed material. The azo-dye coupling method, in addition to reacting with tyrosine, reacts with tryptophan and *histidine* and this seems to be the only test for the latter.

Azo-dye coupling. After downgrading, the slides were placed in a 0·1% solution of fast blue B (the stable tetrazotate of *o*-dianisidine) in either a veronal acetate or borate buffer at pH 9·2 for 5 min. This produces a yellow colour, thought to arise from the coupling of the diazo group at one end of the molecule, to the amino-acid groups in the tissue. That this reaction is genuine was shown by the fact that benzoylation for 16 h, which blocks tyrosine, tryptophan, and histidine, almost completely prevented the reaction. The slides were then washed in buffer solution to remove excess of fast blue, and placed in a 0·1% solution of alpha-naphthol in the same buffer for 2 min. The alpha-naphthol couples on to the second diazo group at the other end of the fast blue molecule, producing a red colour; α-naphthol alone did not stain sections and no reaction was obtained with filter paper (contrast Burstone, 1955). On the other hand, filter paper soaked in tyrosine and tryptophan solutions gave a similar reaction to that above.

In order to make the results more specific, reactions that block certain groups were used before coupling. Tyrosine groups were blocked by the DNB treatment for detecting amino-groups. Tryptophan was destroyed by treatment with peracetic acid for 10 min, and tyrosine and tryptophan were blocked together by treatment with Gram's iodine (Landing and Hall, 1956). The effectiveness of iodine blocking has been questioned by Lillie (1957), who considers that histidine, too, is iodinated and that iodination does not prevent azo-coupling. In the present study iodine treatment prevented reactions with Millon's reagent and DAB in all parts except the fully keratinized fibre. Yellow or brown non-specific staining was observed when isatin was used in attempts to detect proline, ninhydrin followed by chromotropic acid and 2-hydroxy-diphenyl to detect glycine and alanine respectively, and periodic acid followed by chromotrophic acid for serine, even in frozen-dried skin.

Nucleic acids

The methyl green-pyronin method was used for ribonucleic acid (RNA) and Feulgen's method for deoxyribonucleic acid (DNA).

Results (see table 1)

Effect of pH on acidophilia and basiphilia

With orange G at pH 3 most parts stained various shades of orange, the intensity (i.e. the acidophilia) increasing at successively higher levels of the fibre. The intensity of the stain decreased with increasing pH and few parts stained at all above about pH 4. The fully keratinized fibre stained, but with decreasing intensity, up to about pH 6. Odland, on the other hand, found that all parts except his fibrillation zone stained over the whole pH range. The medulla was as dense as the cortex at pH 3, but at pH 5 it had become paler than the cortex.

Indigo-carmine gave similar results; few parts stained above about pH 4. The medulla and inner sheath stained at pH 3, but at pH 4 only the trichohyalin in these parts stained. In contrast to the behaviour with orange G the

fully keratinized fibre and the connective tissue stained over the whole pH range, but with gradually decreasing intensity. There was a slight nuclear stain, as with orange G.

With methylene blue the intensity of the staining increased gradually as the pH increased, but the nuclei (basiphil) stained intensely even at pH 3, and trichohyalin (acidophil) never stained. The cyptoplasm of the bulb cells was

TABLE 1

Summary of Results

The reactions refer to the most vigorous conditions and have been simplified to weak, moderate, and strong; for full description see text

	Bulb	*Fibre*			*Sheath*	
		unkeratinized	*pre-keratinized*	*fully keratinized*	*inner*	*outer*
Acid groups Gram reaction	G−	G−	G+	G+	G+	G−
Amino-groups						
ninhydrin	−	−	−	+	−	−
FDNB	+	+	+	++	+++	++
3-OH	+	−	++	++	+++	+
Arginine	+	++	++	++	+++	+
Tyrosine						
Millon	+	+	++	+++	++	+
nitrosation	+	+	++	++	+	+
azo-coupling after peracetic acid	+	+	+	+++	+++	+
Tryptophan						
DAB	+	+−	+	++	++	+−
NED (Zenker-fixed)	−	−	−	+	+	−
Azo-coupling after FDNB	+	++	++	+++	+++	+
Histidine azo-coupling after iodine	+	+++	+++	+++	+++	+

intensely stained, and, in agreement with Odland, it was found that the stain intensity decreased gradually at successively higher levels of the fibre, the decrease becoming marked between the fibrillary and the fully keratinized regions. Contrary to Odland's results, the fibrils stained as low as pH 3, and in addition to the bulb, cytoplasmic staining at this pH was found in the epidermis and outer sheath. The inner sheath began to stain at pH 4 with a turquoise shade, and the fully keratinized fibre at pH 5, whereas Odland found that this did not stain until about pH 7·5. The cuticle and cortex stained only palely at first, but the medulla stained intensely at once (pH 5). At about pH 6 most parts were stained intensely blue and the intensity increased little if at all above that pH. The stain of the fibre, however, increased only gradually in intensity, not becoming very marked until pH 8.

In a number of instances one half of the cortex was more intensely stained than the other.

Similar results were obtained with basic fuchsin. Again there was a gradual increase in the intensity of staining as the pH increased, but the fully keratinized fibre, medulla, and inner sheath were stained even at pH 3.

Treatment of sections with FDNB and taninol W.R. (I.C.I.) before sacpic produced little change in the result. Benzoylation, however, resulted in the nuclei, streaks in the fibre, and trichohyalin staining red (change from acidophilia to basiphilia). Increased basiphilia has been observed in the fibres of ancient parchment preparations (Ryder, 1958*a*).

Acidic (carboxyl) groups

With Gram's stain, the bulb, fibrillary region, outer sheath, epidermis, and connective tissue were Gram-negative. Those parts normally basiphil were Gram-positive, viz. nuclei, the pre-keratinization region (reaction starting as streaks in the fibrillary region), medulla, and inner sheath. In this, the trichohyalin was Gram-positive, at a higher level only the Henle layer was, then both layers were, and finally at the upper limit of the sheath only the cuticle was positive.

Except for trichohyalin, this result closely follows the usual basiphil result. In addition the stratum corneum, the brushes, and sometimes the vitreous membranes of shedding follicles, were Gram-positive, although the fully keratinized part of the fibre was unstained or yellow (with iodine). Zenker fixation caused the fibres to become slightly Gram-positive, but only above the skin surface (this phenomenon is discussed below). Pre-treatment with peracetic acid for 10 min made the fully keratinized fibre stain strongly positive.

Basic (amino) groups

In fresh-frozen, frozen-dried, formalin-fixed, and Zenker-fixed skin, the only reaction obtained with *ninhydrin* was a pale purple stain in the fully keratinized fibre. This was frequently limited to that part below the skin surface.

Other colours were produced, viz. yellow, the colour ninhydrin gives with proline, in the lower part of the fibre and in the inner sheath. Intensely stained fibres had red and brown shades as granular streaks on the paler purple background. Glycine is known to give a reddish-purple colour and certain peptides a brown colour. Strong ninhydrin solutions are known to give brown shades, but dilution of the solution to 50% with absolute alcohol did not change the result.

In some fibres, particularly in the frozen-dried skin, the cuticle and outer parts of the cortex remained unstained. In other fibres only the parts near the cut ends had stained densely; the central, more weakly stained part was asymmetrical. This supports the suggestion that asymmetrical staining is due to differences in accessibility.

FDNB gave a general yellow colouring that was somewhat indefinite under the microscope. Fresh-frozen, frozen-dried, and Zenker-fixed material all gave a stronger reaction than formalin-fixed material, no doubt owing to the reaction of formalin with amino-groups. The bulb, fibrillary, and pre-keratinization regions were pale; the connective tissue, epidermis, outer sheath, and fully keratinized fibre all reacted moderately, although in thick frozen sections the fibre was negative or pale. The inner sheath gave the strongest reaction and the nuclei, in general positive, were apparently negative in the bulb.

The colour obtained with *3-hydroxy-2-naphthaldehyde* varied from maroon to purple in different slides, probably owing to unintentional variations in pH. Zenker-fixed material gave a stronger reaction than did frozen-dried, probably owing to the release of groups by the fixative, the most marked difference being in the connective tissue, which reacted slightly to moderately. The bulb, outer sheath, and epidermis were pale and their nuclei were a little more densely stained. In Zenker-fixed material, however, only the nucleoli and nuclear chromatin of the bulb nuclei reacted, but did so densely. The inner sheath and medulla gave the strongest reaction, but in only the frozen-dried material was the trichohyalin clearly stained. The lower part of the fibre was negative, staining yellow with the fast blue used in the second stage of the reaction. The fully keratinized part gave a moderate to strong reaction in Zenker-fixed material, but was negative or pale in frozen-dried skin. The reaction started at about the pre keratinization level and was denser above the skin surface than below. This was so with most tests on Zenker-fixed material, but it was realized that the fixative had been able to attack the fibre exposed above the skin surface more readily than that below. Asymmetrical staining was observed below the surface but not above. Stratum corneum was often negative (yellow) or palely stained.

Amino-acid residues

Arginine (fig. 1, A). A weak to moderate reaction was obtained in the bulb and outer sheath and the nuclei reacted more strongly. The fully keratinized fibre was negative, but the unkeratinized part gave a moderate reaction. The epidermis reacted moderately to strongly; the medulla and inner sheath reacted strongly and in these parts the trichohyalin gave the strongest reac-

FIG. 1 (plate). A, strong reaction for arginine in the trichohyalin of the inner sheath and medulla with Sakaguchi's method.

B, thick frozen section showing reaction for tyrosine with Millon's reagent in the keratinized fibre only where the reagent has gained access from the unkeratinized part below, and the cut end above.

C, D, and E, preparations coupled with α-naphthol and fast blue B.

C, after treatment with peracetic acid, showing gradual increase in intensity as the fibre becomes keratinized.

D, after treatment with FDNB and showing a reaction in the unkeratinized part of the fibre, paler pre-keratinization zone, and negative keratinized part.

E, after iodine treatment, showing an intense reaction in the keratinized part as well as in much of the unkeratinized part.

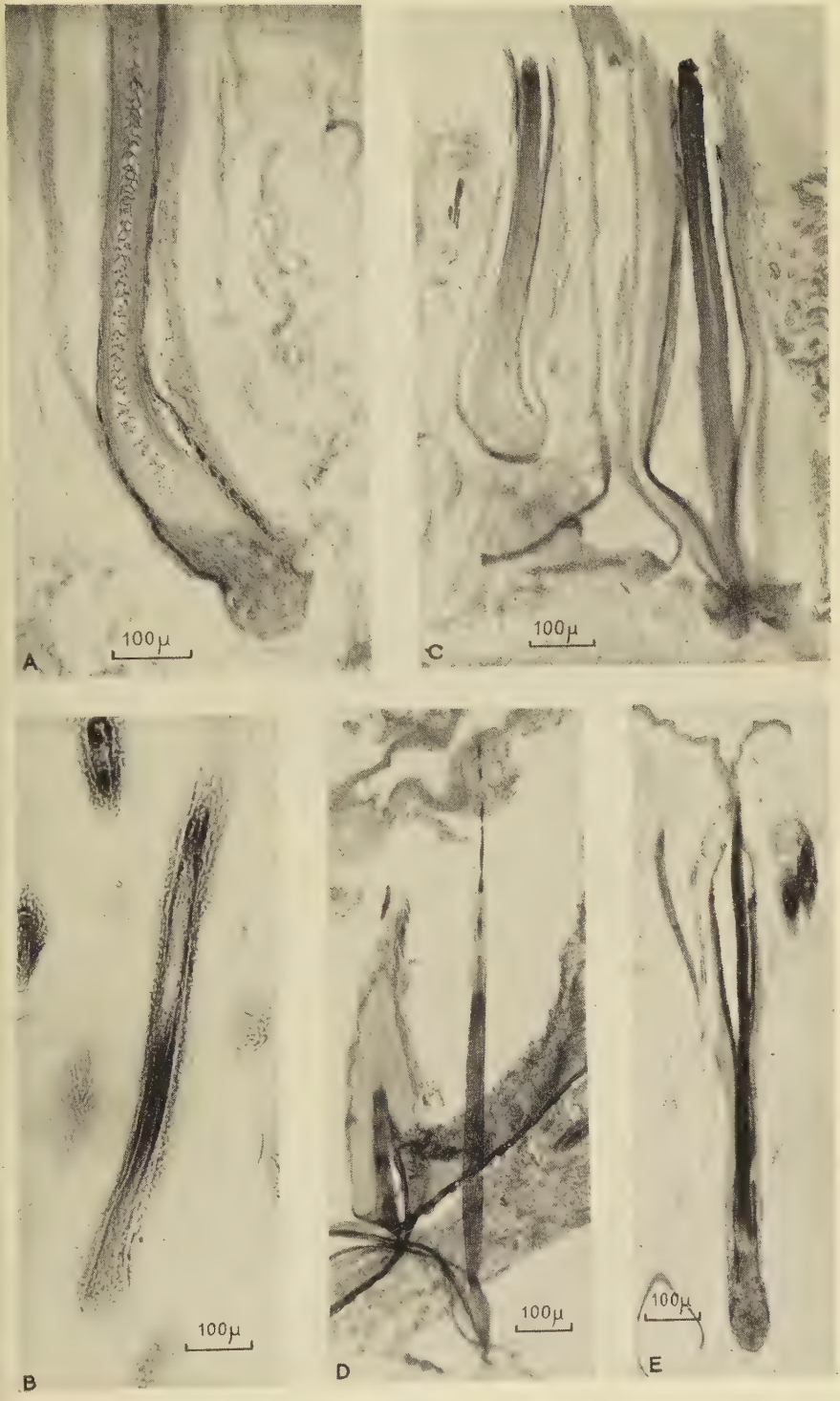

FIG. 1

M. L. RYDER

ion; when trichohyalin disappeared at higher levels, the arginine reaction was educed. Zenker-fixation, and pre-treatment of formalin-fixed skin with eracetic acid for 10 min, increased the reaction in all parts, as well as producng a reaction in the fully keratinized fibre. Frozen-dried skin gave a similar, ut on the whole less intense, reaction. The unkeratinized part of the fibre, owever, gave the strongest reaction, and sometimes, in all types of preparaion, the pre-keratinization region was the only part that had stained.

Tyrosine. In thick frozen sections treated with *Millon's reagent* (fig. 1, B) he bulb and outer sheath gave a very pale reaction and the nuclei were egative. The epidermis and inner sheath gave a weak to moderate reaction, Henle's layer being only slightly more intense than Huxley's. The reaction f the fibre increased in intensity at successively higher levels, starting noderately in the fibrillary region. The cuticle and cortex stained an intense ut-brown, but in thick frozen sections containing whole fibres, the reaction nly occurred near the cut ends where the reagent could gain access. The eaction was either entirely on one side of the fibre (on the outside of the urve of the crimp) or denser on that side than the other. The medulla was ither negative or gave a weak reaction. The connective tissue was virtually egative, but the glands gave a weak reaction. Thin sections gave similar eactions, but the intensity was stronger in fixed skin, and weaker in frozenried skin. However, because the fibres were now sectioned, the whole of the ully-keratinized part gave a reaction, but the bilateral staining was less narked.

The maroon-purple reaction obtained after *nitrosation* in general paralleled hat obtained with Millon's reagent, except that the nuclei gave a strong eaction and the connective tissue was moderately dense. There was the same ncrease in intensity at higher levels of the fibre, but with some evidence that he strongest reaction was in the pre-keratinization region. The reaction in the ully keratinized fibre was weaker than with Millon's reagent (and sometimes ellow, compare NED below), and no asymmetry was observed, but the esults of most tests in the fibre seem to be dependent on the vigour of the eaction. Both epidermis and inner sheath were weaker than with Millon's eagent, whereas the latter is often the part that gives the strongest reaction.

Tryptophan. This seems to be easily destroyed by acid; no reaction was btained with DAB in Zenker-fixed material, or in skin that had been stored n unbuffered formalin for several years. Skin recently fixed in buffered ormalin gave a general blue reaction with DAB. The cytoplasm of the bulb as pale to moderate, and the fibrillary and pre-keratinization regions were ery pale. The fully keratinized fibre reacted a little more strongly, but one alf (inside the curve) gave hardly any reaction, and the medulla was paler or egative. The Henle layer of the inner sheath was densest with a moderate eaction, Huxley's layer was pale or negative. The outer sheath and epidermis ave hardly any reaction, and the connective tissue was negative. Sweat lands and muscles reacted moderately and red blood-corpuscles intensely.

The nitrosation method for tryptophan produced a general yellow-brown

colouring which was changed to purple by the subsequent coupling with NED. Except at cut ends, the fully keratinized fibre remained yellow, in fresh-frozen and in parts of the frozen-dried skin. In Zenker-fixed skin the whole length of the fibre became purple, but showed clear asymmetry. In Zenker-fixed skin, the fully-keratinized fibre and the inner sheath were the only parts giving an appreciable reaction, and in view of the negative reaction in Zenker-fixed skin with DAB, and the fact that a similar method is used for tyrosine, it is concluded that this method might not be specific for tryptophan. The results of tests on filter paper soaked in solutions of the amino-acids were inconclusive.

Azo-dye coupling for tyrosine, tryptophan, and histidine. Treatment with fast blue B produced a yellow reaction of varying intensity in different parts of the skin and follicle; the connective tissue was palest, and the inner sheath darkest. Except for the medulla the fully keratinized part gave very little reaction, even in thin sections, and the fibrillary region, too, was occasionally colourless. Thick-frozen and thin wax sections (both fixed and frozen-dried) gave results that were alike, but as with other tests fixed skin gave the strongest reaction.

Subsequent coupling with α-naphthol produced a red colour, the intensities of which paralleled the intensities of the original yellow reaction. Parts that had been colourless in the yellow stage were, of course, still colourless, but in one instance in frozen sections the fibrillary region remained yellow. This could not be repeated, but it suggested steric prevention of the α-naphthol from reaching the second diazo group of the fast blue, or that both diazo groups might have coupled on to the tissue and even cross-linked two of the long-chain molecules of the fibre.

The bulb was maroon, and in the fibrillary region, the fibrils stood out moderately red on a paler background. The pre-keratinization region was paler, and the fully keratinized part gave either no reaction, or a streaky, pale-red reaction; the medulla was moderately intense. In thick-frozen sections containing whole fibres, the cortex gave a moderately intense reaction only at the cut ends. The inner sheath reacted most intensely, the Henle layer being more intense than the Huxley layer (compare some of the amino-acid reactions, e.g. DAB). Alternate sections showed that the more intense coupling reaction coincided with the red stain of sacpic, but trichohyalin was indistinguishable in coupled preparations. The outer sheath was pale red, the nuclei being deeper; the epidermis was deep red, the stratum corneum being most intense. Connective tissue was either orange or medium red.

Results of coupling after blocking. The three blocking reactions were compared with each of the other blocking reactions, in pairs, with the use of alternate sections. They were also compared with the specific methods, e.g. Millon's reagent was compared with coupling following peracetic acid treatment, which destroys tryptophan. Although pre-treatment with peracetic acid made the coupling reaction generally weaker, in contrast to the reaction in unblocked preparations there was an intense maroon reaction throughout the fully keratinized fibre, and no asymmetry was obvious (fig. 1, c).

Exposure to FDNB, which blocks tyrosine, reduced the overall intensity of the coupling reaction, but not as much as did peracetic acid. The fully keratinized fibre was negative or had a streaky, pale-red reaction often on one side only, although the medulla reacted moderately (fig. 1, D) (negative with DAB). The pre-keratinization region was pink, and there was a tendency for the reaction below this to be stronger compared with unblocked preparations. This does not agree with the almost negative reaction with DAB in the lower part of the fibre.

A suspicion that the FDNB had not really blocked the reaction in the fibre, because it had not been able to penetrate, was confirmed in Zenker-fixed material in which the fixation must have made the groups accessible. In this there was a strong coupling reaction after treatment with FDNB, but only in that part above the skin surface. When sections were treated with iodine to block both tyrosine and tryptophan, they became orange instead of yellow after the first coupling with fast blue. Coupling with α-naphthol changed the colour to maroon and the result was more intense than in unblocked preparations. Both the fully keratinized and lower part of the fibre gave an intense maroon reaction (fig. 1, E).

Nucleic acids

There was an intense reaction for RNA in the lower part of the fibre, which terminated asymmetrically at about the pre-keratinization level. This was prevented or considerably reduced by pre-treatment with perchloric acid. The concentration of RNA here might be associated with protein synthesis. There was, in addition, a strong RNA reaction in the bulb, but much less in the outer and inner sheaths. All nuclei normally evident with sacpic stain gave a DNA reaction. Those in the papilla and lower half of the bulb reacted most intensely, and the reaction in the fibre and inner sheath disappeared on keratinization. Both results agree with the observations of Hardy (1952).

Discussion

Auber (1952) found that picric acid was the only acid dye that would stain the fibre under normal conditions, so he regarded the phenomenon as 'nitrophilia' rather than acidophilia. In fact picric acid has a very generalized dyeing action which may be due to its small molecular size allowing penetration of spaces that are barred to larger molecules.

The investigation of the effect of pH on staining in the present study threw light on the interpretation of staining reactions, but it is doubtful whether one can determine isoelectric points in this way because of overlap in the reactions of acid and basic dyes. However, the value of about pH 5·0 for wool agrees with the accepted figure. The dyes behaved as expected: acid dyes dyed more readily in acid solution, and basic dyes more readily in alkaline solution. Thus the results obtained with orange G were more easily explained than the staining over the whole pH range found by Odland (1953). But the difference might be due to differences in the stain and the material.

That nuclei are strongly basiphil even in acid solution accords with the results from ordinary staining. But the basiphilia, even in acid solution, of the lower part of the follicle, and its decrease as keratinization takes place, does not accord with the acidophilia of the lower part, and the basic staining of the pre-keratinization region with sacpic. The acidophilia in the lower part is in keeping with the lack of basic groups, and the basiphilia of the pre-keratinization region with the Gram-positive reaction for acidic groups.

There is a possibility that the reaction with ninhydrin in the fully keratinized fibre is due to non-specific staining; when ninhydrin reacts with an α-amino-group the coloured compound formed is split off from the tissue and could diffuse away to stain other parts. However, the other reagents for amino-groups gave a reaction in the fibre, and it may be that here the compound formed (which has a molecule twice the size of the original ninhydrin) is enmeshed within the fibre and is unable to diffuse away. This is supported by the fact that pre-treatment with FDNB prevents the ninhydrin reaction.

It is interesting that parts which are normally basiphil gave a Gram-positive reaction. However, the intensity was variable, probably owing to the differentiation, which is a disadvantage of the method (Pearse, 1953). When the pre-keratinization region and the inner sheath were strongly positive, the connective tissue was weak (compare Pearse); and when the former were weak, the latter was negative. Such variation may possibly arise from variations in the 'polymerization' discussed by Pearse.

The Gram-positive reaction of the acidophil trichohyalin was unexpected, but it is associated with the general Gram-positive reaction of the inner sheath, which was very reactive with most reagents used. Rogers (1958) found that the inner sheath was rich in both acidic and basic amino-acids. The acidophilia of trichohyalin accords with the intense reaction it gives for amino-groups and also with the reaction for the basic amino-acid arginine.

With arginine we begin to consider the interpretation of the reactions for specific amino-acid residues in the light of the striking variations obtained with different prior treatments. With no previous knowledge one could have been led, from the use of formalin-fixed material, to believe that the fibre contained no arginine; it only became evident here after vigorous treatment, for instance, with Zenker fixation or with peracetic acid, which no doubt exposes groups to react that were previously not accessible.

FDNB would not be expected to penetrate the keratinized fibre to the same extent as peracetic acid, which in addition to destroying tryptophan breaks disulphide bonds. Its behaviour therefore shows that a negative result must not be taken as indicating the absence of groups, neither does the intensity of the reaction necessarily indicate the amount present, but rather the availability of the groups to react in the conditions used. For example, whereas the general pale reaction for tryptophan and the moderately intense reaction for tyrosine agree with the figures of about 1% of tryptophan and 5–6% of tyrosine in wool, 9–10% of arginine is associated with only a moderate reaction, and 1% histidine with an intense reaction. The generally paler

eaction with frozen-dried skin is interesting because it suggests that histo-ogically proteins normally have few groups available to react and even a mild xative such as formalin releases them.

There was little difference in distribution between the different amino-cids. Keratinized parts of the fibre and inner sheath gave the strongest reac-ion. This is comparable with the concentration of sulphur in the fibre on eratinization (Ryder, 1958*b*). The explanation for the intense reaction ob-ained by coupling after treatment with iodine is unknown. That in the fibre ould have been contributed to by incompletely blocked tyrosine and trypto-han, but that in the lower part would seem to be histidine only, although his should also have reacted in the preparations treated with FDNB and eracetic acid.

This investigation has thrown light on the now well-known bilateral nature asymmetrical staining) of the fibre, which has been attributed to chemical ifferences between the two halves. It was not always possible to tell which alf gave the reaction; with Millon's reagent it was on the outside of the curve f the crimp (the orthocortex) and with the DAB reaction it was on the inner ide (the paracortex). But all the evidence from this investigation suggests hat the phenomenon is associated with accessibility rather than with a uantitative chemical difference between the halves: asymmetrical staining ave way to uniform staining after vigorous treatment, and asymmetry was ar less marked in thin sections. A difference in accessibility may arise from purely physical swelling of one half (Kassenbeck and Leveau, 1957) and not ecessarily from a difference in the chemical availability of groups.

I wish to thank Dr. A. B. Wildman for encouragement with this work, Mr. G. R. Standley and Miss M. M. Vickers for technical assistance, Mr. G. R. Lee for help with the preparation of certain reagents used, and Mrs. G. M. Probert for taking the photomicrographs.

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An Electron Microscope Study of a Small Free-living Amoeba (*Hartmanella astronyxis*)

By K. DEUTSCH *and* M. M. SWANN

(*From the Department of Zoology, University of Edinburgh*)

With two plates (figs. 1 and 2)

Summary

The fine structure of a species of small free-living amoeba, *Hartmanella astronyxis*, has been investigated. The mitochondria resemble those of other species of amoeba. Structureless bodies of about the same size as mitochondria are sometimes found in association with them. Double membranes are common in the cytoplasm, and may show granules along their outer borders. The nuclear membrane is a double-layered structure, with a honeycomb structure evident in tangential sections. The cell membrane is also double-layered, or occasionally multi-layered.

Introduction

A NUMBER of electron microscope studies have been made on *Amoeba proteus* and *Chaos chaos* (Andresen, 1956; Bairati and Lehmann, 1951, 1954; Cohen, 1957; Greider, Kostir, and Frajola, 1956; Lehmann, Manni, and Geiger, 1956; Manni, 1956; Pappas, 1956; Rudzinka, 1956; Sedar and Rudzinka, 1956). Other amoebae, however, seem to have been neglected. *Hartmanella astronyxis*, a small species of soil amoeba, originally isolated by Ray and Hayes (1954), has recently been maintained in this laboratory in sterile culture. Since it is being used for a number of studies of cell growth and cell division, we felt it desirable to make a general study of its structure. The results of this work are presented below. The results of a more detailed investigation of the changes during mitosis will be presented in due course.

Material and Method

The amoebae were fixed in 1% osmium tetroxide (or 10% formalin) at pH 7·2. The fixed amoebae were then embedded in a methacrylate mixture (93% butyl methacrylate, 7% methyl methacrylate), or in a methacrylate mixture to which tetra-ethyl tin had been added (4 parts butyl methacrylate, 1 part methyl methacrylate, 0·5 parts tetra-ethyl tin). It was originally hoped that the addition of tetra-ethyl tin to the embedding medium would render it dense enough to make the specimens appear light on a dark background (Deutsch, 1957); but in this we were disappointed, since it proved impossible to dissolve a sufficient quantity of tin in the methacrylate mixture. However, some parts of the tissue were apparently 'stained' by the tin, and we have included a few photographs of sections embedded in this medium. The possibilities of the method are being further investigated.

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Sections were cut at 250 Å, and examined in a Siemens Elmiskop I, at magnifications of 8,000 and 40,000. The plates were enlarged photographically.

Results and Discussion

A low power view of the amoeba is shown in fig. 1, A. The nucleus, nucleolus, food vacuoles, and mitochondria are readily visible.

The nucleoplasm is mainly granular in osmium-fixed sections (fig. 1, B), while the addition of tin to formalin-fixed specimens gives a rather more vesiculate appearance (fig. 1, C). The nuclear membrane consists of two electron-dense layers enclosing an electron-transparent one, the width of each layer being about 70 Å (fig. 1, G). This double structure is most clearly seen in sections 'stained' with tin (fig. 1, F). Tangential sections of the nuclear membrane (figs. 2, D; 2, F) show either a 'honeycomb' organization, the diameter of the pores being about 50–100 Å, or a vesiculate appearance (compare Greider, Kostir, and Frajola, 1956; Pappas, 1956). The nucleolus is very electron-dense, and usually contains a reticulated inner region (fig. 1, B).

The cytoplasm contains many of the structures normally encountered in electron micrographs. The mitochondria (fig. 2, B) resemble those found in other Protozoa (Manni, 1956; Sedlar and Rudzinka, 1956), containing numerous double membranes, somewhat contorted. There are food vacuoles and a large number of small vesicles (fig. 1, A). There are also double membranes scattered throughout the cytoplasm, often with small dense granules arranged along their outer borders (figs. 2, C; 2, E; compare the endoplasmic reticulum of Palade (1955)). We have not, however, found any trace of a Golgi apparatus.

A few cells show a less usual form of inclusion, about the same size as the mitochondria, but devoid of internal structure (see figs. 2, G; 2, H; compare Rouiller and Bernhard, 1956). It is our impression that these bodies are only found in cells near to mitosis, and that they may be in some way associated with the mitochondria (see arrows in figs. 2, G; 2, H).

The cell membrane consists either of two electron-dense layers enclosing an electron-transparent one (figs. 1, D; 1, E) or, less often, of a number of alternating dense and transparent layers (fig. 2, A). The layers are about 70 Å thick.

We are indebted to Mr. A. E. G. Dunn for his most skilful assistance, and to the Melville Trust for Cancer Research who provided the microscope.

Fig. 1 (plate). A, low-power view of *H. astronyxis*, showing nucleus, nucleolus, food vacuoles small vesicles, and mitochondria. (Osmium.)

B, nucleus, showing nucleolus with reticulated centre. (Osmium.)

C, nucleus, showing vesiculate appearance when fixed with formaldehyde and 'stained' with tin.

D, cell membrane showing double structure. (Osmium.)

E, cell membrane showing double structure. (Formaldehyde, 'stained' tin.)

F, nuclear membrane, showing double layer. (Osmium.)

G, nuclear membrane, showing double layer. (Osmium, 'stained' tin.)

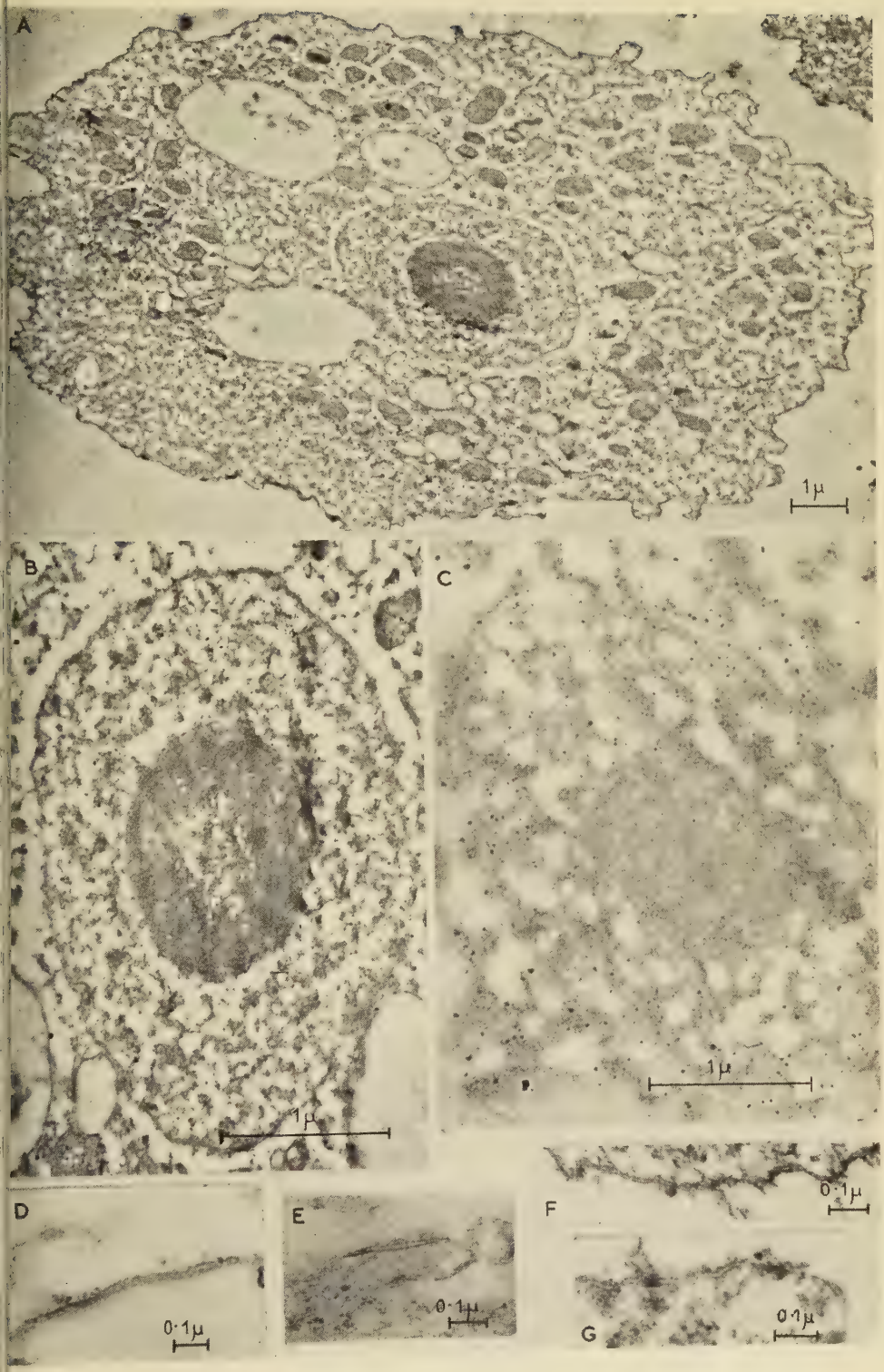

FIG. 1

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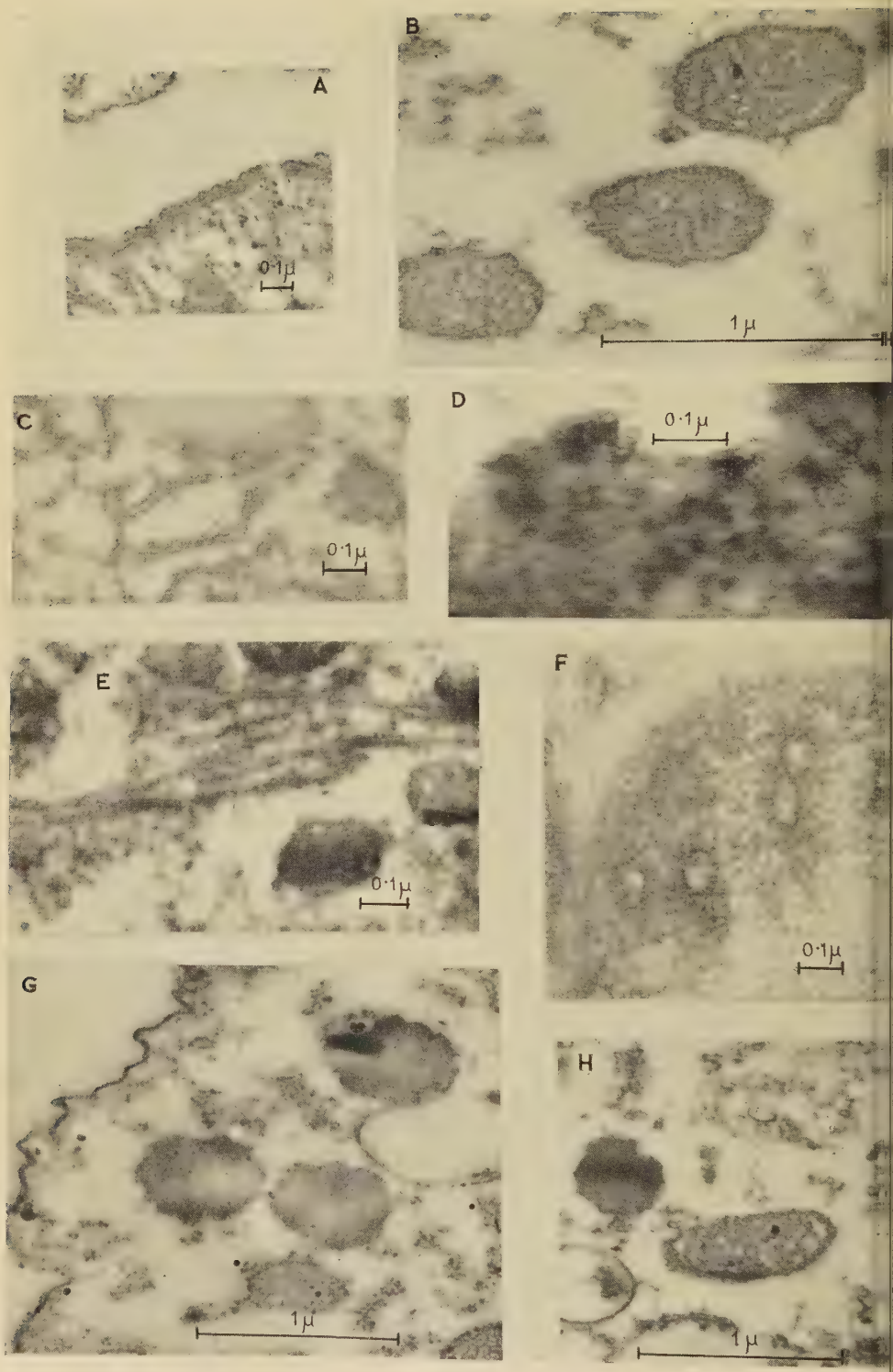

FIG. 2

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FIG. 2 (plate). A, cell membrane, showing multi-layered structure. (Osmium.)
B, mitochondria, showing double membranes. (Osmium.)
C, cytoplasmic double membranes, with granules along their outer border. (Osmium.)
D, nuclear membrane in tangential section, showing honeycomb structure. (Formaldehyde, 'stained' tin.)
E, cytoplasmic double membranes, with granules along their outer border. (Osmium.)
F, nuclear membrane in tangential section, showing vesiculate appearance. (Osmium.)
G, structureless bodies and mitochondria. Note the apparent association between the two. (Osmium.)
H, structureless bodies and mitochondria. Note the apparent association between the two. (Osmium.)

A Phase Contrast Study of the Blood-cells in *Prodenia* Larvae (Order Lepidoptera)

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Summary

The various classes of blood-cells in air-dried, Wright-stained blood-smears described by Yeager (1945) from heat-fixed *Prodenia* can be recognized in unfixed and unstained preparations with the phase microscope. With only one exception, the blood-cells do not undergo extensive transformations *in vitro* for at least one hour. Hence, the blood-cells may be accurately identified and differentially counted by phase microscopy. Two kinds of blood-cells undergo marked alterations in appearance on heat-fixation, whereas the other types remain mostly unmodified. One type of blood-cell, the oenocytoid, typically undergoes a marked change in character *in vitro* in unfixed blood. Several changes in Yeager's classification of insect blood-cells are proposed, and it is suggested that this new system will be found sufficient for identifying and comparing blood-cells in the different orders of insects.

Introduction

YEAGER (1945) found many different kinds of blood-cells in the southern army-worm larva (*Prodenia eridania* Cram.) after fixing the insects in hot water (60° C for 5 or 10 min) and staining air-dried films with Wright's stain. He used heat-fixation because he felt that the blood-cells would undergo extensive transformations (become what he terms 'active' forms) *in vitro* if unfixed. He adopted the term 'active' from Fauré-Fremiet (1925). Yeager assumed that heat-fixed blood-cells retained the form they possessed in the circulating blood. At that time he did not have a method for adequately studying the cytological details of unfixed cells, although he made a number of important observations on unfixed blood-cells with the ordinary light microscope.

The present writer found that unfixed, unstained blood-cells of both *Tenebrio* (Jones, 1954) and *Sarcophaga* (Jones, 1956) could be readily identified and differentially counted with the phase microscope. It seemed, therefore, desirable to determine if unfixed blood-cells from the southern army-worm could also be identified by phase microscopy and to see what cytological changes took place *in vitro* and to contrast these with alterations after the animals had been heat-fixed.

Materials and Methods

Only rather large larvae, 25 to 30 mm in length, which had been fed on fresh kale, were used for these studies. Unfixed blood was obtained from living larvae by severing a proleg and allowing a drop of blood to exude on to a clean glass slide. A coverslip, either unringed or previously ringed with white

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petrolatum, was gently placed on top of the blood and the wet preparation then examined under oil immersion at magnifications of 800 to 1,250. A stop-watch was started immediately after cutting the proleg so that an accurate record could be kept of changes occurring with time. To obtain heat-fixed blood, the entire larva was then immersed in a water bath at 60° C for 1 to 10 min. The animal was then removed from the water and dried. A proleg was severed to make a wet coverslip preparation as described above.

Results

Unfixed blood from large *Prodenia* larvae is pale green and very slowly turns either dark brown or jet black in a moist chamber. The darkening of the blood may be greatly accelerated by treating it with a small amount of ethanol, just sufficient to cause a white precipitation in the plasma. Untreated plasma does not coagulate. When larvae are heat-fixed at 60° C for one min, the blood is seen to have a very pale yellow cast and does not blacken within 24 h.

A thin coverslip mount of fresh, untreated *Prodenia* blood, examined under oil immersion with a phase microscope, reveals that most of the blood-cells undergo strikingly few cytological modifications for at least one hour after withdrawal. All of the cell classes which Yeager (1945) described in heat-fixed, Wright-stained blood smears can be readily identified. The phase microscope makes apparent many hitherto unknown details concerning several blood-cells.

In unfixed blood, the plasmatocytes and plasmatocyte-like cells (proleucocytes (= prohemocytes), smooth contour chromophil cells, and vermiform cells) have various amounts of dark to pale grey cytoplasm (fig. 1, A–J, L) that contains either thin filamentous or small non-refringent granular mitochondria, usually congregated around a single large nucleus. The nucleus has separate, mostly evenly distributed granules of chromatin and one or two larger intranuclear masses. In unfixed preparations the plasmatocytes and plasmatocyte-like cells either (*a*) retain their original shape (round, ovoid, spindle, vermiform); (*b*) retract their spindle ends and thus partially round up (vermiform cells do not retract their spindle ends); (*c*) send out either thin filamentous or delicate blade-like cytoplasmic extensions; or, less commonly, (*d*) spread out excessively and disintegrate.

While plasmatocytes and cells closely related to them have a marked tendency to clump together in unfixed blood (vermiform cells do not clump or stick to other cell classes), many of them still remain quite free and separate. After heat fixation the blood-cells do not clump together conspicuously and the plasmatocytes and related cells appear larger and retain their spindle ends. Their fine cytoplasmic filaments can no longer be made out with clarity under the phase microscope.

Unfixed podocytes (cells with long, often equal, permanent cytoplasmic extensions, fig. 1, K) although obviously closely related to plasmatocytes, differ from them in several important particulars. Podocytes do not undergo any changes in general shape for at least an hour in unfixed coverslip prepara-

tions. They are always exceedingly flat cells with 3 to 8 pointed processes or cusps. Podocytes do not usually stick to other blood-cells and usually float at the uppermost portion of wet mounts. Their pointed extensions are flexible and sometimes waved about if the plasma is caused to flow past them rapidly. Podocytes lose much of their delicacy after heat-fixation.

In untreated blood from *Prodenia* larvae a number of thick, usually small blood-cells are seen, filled with large, dark grey, rounded or ovoid cytoplasmic inclusions surrounding and generally obscuring a small central or slightly excentric nucleus (fig. 1, Y–AD). Although Yeager (1945) termed these cells 'eruptive', they were very rarely observed to 'erupt' in unfixed blood for at least an hour. Furthermore, unfixed cells did not 'erupt' even after deliberate pressure had been applied to the coverslip with the objective. After heat-fixing larvae for only one minute at 60° C, these cells are often found to have undergone a marked alteration in appearance, for the large cytoplasmic spherules are no longer dark grey masses of dense material but appear as open vacuolar areas (fig. 1, AD). Sometimes a small black granule remains in one or two vacuoles and these may oscillate within the vacuoles. The cells, after heat-fixing, are often distorted and tend to disintegrate rapidly. In larvae which are about to pupate some of these cells in unfixed blood contain 'empty' spherules (vacuoles) and also pale grey and dark grey spherules. This suggests that material within them is being delivered into the plasma (fig. 1, AC), but even in these cells eruption does not normally occur. The cells with spherules, unlike other blood-cells, appear to rock back and forth slightly where no plasmal currents are evident. Possibly this movement represents a type of locomotion; it appears to be independent of the condition of the spherules.

Large round or ovoid cells filled with numerous, distinct, bright granules of medium size are seen in unfixed wet mounts (fig. 1, U–X). In these granular cells the large round nucleus is excentric and often totally obscured. On rare occasions these cells will send out either very thin filaments or large blade-like extensions of pale grey cytoplasm, but most of them preserve their round or ovoid shape *in vitro*. When the larvae are fixed by heat the granular blood-cells undergo profound cytological changes. Distinct, grey, non-granular pointed ends are then visible at both poles of a very large rounded cell-body. The heat-fixed cell appears bloated with large, ill-defined, round, pale grey to clear vacuole-like masses, which totally obscure the nucleus. The cells in wet mounts partially disintegrate within a short time: large grey or clear bubbles appear at the edges of the cells. Yeager (1945) termed these heat-fixed cells 'cystocytes' (but see table 1 of this paper for suggested terminology).

In unfixed prepupal *Prodenia* blood, round cells of medium size, with a dimly visible nucleus and with several small, round, bright, fat-like droplets in the cytoplasm become conspicuous (fig. 1, Q–T). Their cytoplasm contains several different types of granular inclusions as well. These cells tend to send out filaments of grey cytoplasm. Eventually these cells become completely filled with large, round, mostly non-refringent inclusions and they then closely resemble the granular blood-cells described in the preceding section. They differ

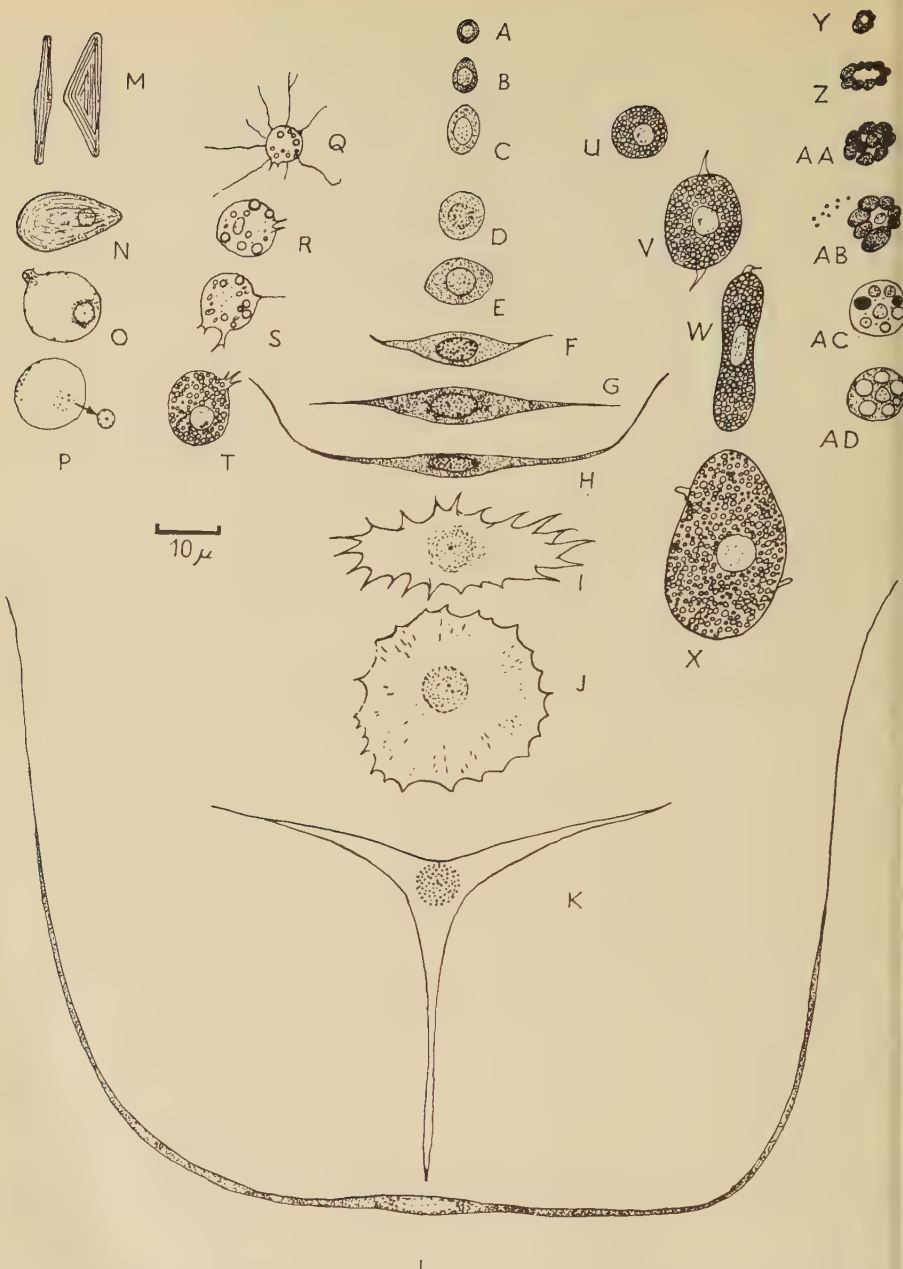

FIG. 1. Appearance of unfixed blood-cells of *Prodenia* larvae. All somewhat schematized. A, prohaemocyte (plasmatocyte-like cell). B, transitional cell (plasmatocyte-like cell). C–E, round or ovoid plasmatocytes. F–H, spindle and elongated plasmatocytes. I, plasmatocyte with cytoplasmic extensions ('active' form). J, greatly spread out plasmatocyte, just before disintegration. Note thread-like mitochondria. K, podocyte. L, vermiform cell (plasmatocyte-like cell). M, oenocytoid immediately after removal of blood. N, oenocytoid just beginning to transform. O, fully transformed ('active') oenocytoid. P, oenocytoid losing its nucleus. Q, spheroidocyte with thread-like extensions. R–T, various stages of spheroidocytes. U–X, various forms of granular haemocytes. Y–AA, typical spherule cells. AB, intact spherule cell which has just released droplets into plasma. AC, spherule cell with full, partially empty, and completely empty spherules. AD, completely empty spherule cell.

from the latter, however, in remaining essentially unmodified after heat-fixation. Yeager (1945) referred to those cells as spheroidocytes but incorrectly thought the inclusions were vacuoles. Presumably the granular inclusions in these cells were dissolved by the methanol in the staining procedure he employed.

Only one kind of blood-cell in *Prodenia* tends to be very unstable in unfixed plasma. This triangular or elongate-ovoid cell has very smooth, dark grey cytoplasm containing most strikingly aligned filamentous inclusions around an excentric and dimly defined nucleus (fig. 1, M, N). Long thin strands of what appear to be very fine dark granules complexly folded into lamellated strands can be seen within the cytoplasm for only a brief time in unfixed blood. The nucleus rapidly becomes clearer as the strands of granules suddenly disappear, and simultaneously the cytoplasm grows crystal clear (fig. 1, O). Many fine bright round granules cluster around the nucleus and at the rim of the cell. Often the granules undergo violent Brownian movements within the cell. The cell rounds up during this remarkable transformation. Sometimes the nucleus slips out of the cytoplasmic envelope, leaving behind a distinct, pale grey zone filled with very fine dancing particles (fig. 1, P). After their transformation these cells do not then disintegrate but remain thus changed for a long time. Heat-fixation of the larva completely prevents this transformation and preserves the granular strands. Yeager (1945) identified these cells as oenocyte-like cells. He noted that their '. . . cytoplasm may have a texture suggesting a condition of stress and strain or included crystals or rods not readily visible to the eye'.

On the basis of the observations with the phase microscope on unfixed *Prodenia* blood-cells it is suggested that Yeager's (1945) terminology for two of them is unsuitable. It is possible by changing several of his terms to rectify a considerable portion of the confusion that has arisen from various modifications of his classification for different insects. These suggested changes in terminology are presented in table 1.

Discussion

This study has shown first that untreated blood-cells of *Prodenia* larvae do not undergo sufficient transformation *in vitro* to prevent accurate identification of different groups. This means that blood-cells may be studied from living larvae and that more than one differential count can be obtained from single individuals treated in various ways. It has also been shown, by comparison with living cells, that heat-fixation for even a brief period causes separate granules to change into large, ill-defined vacuoles in granular haemocytes, and tends to lead to the premature loss of material from the spherule cells. On the other hand, heat-fixation apparently partially preserves the living appearance of the other kinds of blood-cells, though it does so for only a brief period.

In vitro transformation of blood-cells is a most useful criterion for classifying them, since it establishes the relationships between the so-called 'passive' and 'active' forms. An excellent example of this is the striking *in vitro* transformation of the oenocytoid.

TABLE 1

Yeager's terminology	*Proposed terminology*
Proleucocytoids	Prohaemocytes (plasmatocyte-like cells)
Smooth-contour chromophilic cells	Transitional cells or chromophils (plasmatocyte-like cells)
Oenocyte-like cells	Oenocytoids
Plasmatocytes	Plasmatocytes
Podocytes	Podocytes
Vermiform cells	Vermiform cells (plasmatocyte-like cells)
Cystocytes	Granular haemocytes
?	Cystocytes. Blood-cells which rapidly round up and become brilliantly hyaline and form peripheral blisters *in vitro*. Typically become surrounded by granular clouds. Termed 'coagulocytes' by Grégoire and Florkin (1950). When heat-fixed and stained these cells have many round eosinophil inclusions in an amphophil cytoplasm (Jones, 1950).
Spheroidocytes	Spheroidocytes
Eruptive cells	Spherule cells
Degenerating cells	(Degenerating cells. While a record should be kept of degenerating cells in differential counts, this category does not represent a separate type of cell.)

Yeager (1945) classified the 10 classes of *Prodenia* blood-cells into 32 different types and even more variants for descriptive purposes (Yeager, personal communication). It is a most laborious task to make differential counts of so many different types of cells; and since such counts are often highly variable even within a single smear, it is unprofitable in most cases to do so, especially where it is desirable to make many differential counts. It is obvious that by grouping Yeager's plasmatocytes with other closely related forms (prohaemocytes, chromophils, and vermiform cells), differential counts can be made more easily and rapidly.

Several important but not radical changes are proposed in the terminology of blood-cells of insects. Yeager's 'cystocytes' should be termed *granular hemocytes*, his 'eruptive cells' should be termed *spherule cells*. It is finally suggested that a term *cystocyte* be tentatively retained to describe a kind of blood-cell (not present in *Prodenia*) which initiates coagulation of the blood plasma in the meal-worm (Jones, 1954) and the cockroach (Jones, 1957).

By using the above proposed terminology it should be possible to compare profitably the blood-cells of insects from various orders. The present writer plans to make such a comparison in a review of the morphology and physiology of insect blood-cells.

I wish to acknowledge my great indebtedness to Dr. J. Franklin Yeager for help, advice, criticism, and inspiration in this and other studies of insect blood-cells. This paper constitutes Scientific Article No. A711, Contribution No. 2942, of Maryland Agricultural Experiment Station.

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A Quantitative Study of the Cytoplasm of Ascites Tumour Cells in Mice

By R. J. KING

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With one plate (fig. 4)

Summary

This paper describes how a combination of microspectroscopy and interference microscopy has been used to investigate the composition of the cytoplasm of fresh ascites tumour cells of mice. The accuracy of the microspectroscopic method will be very dependent on the standard absorption curves used (i.e. results obtained from dilute nucleic acid and protein solutions of known concentration), and particular attention has therefore been given to this aspect of the investigation. Thus, the standard protein absorption curve has been derived in two ways, first from a certain assumed amino-acid composition, and secondly by a method involving the optical density and refractive index of the ascitic fluid itself. Both methods are shown to result in similar cellular analyses.

The cytoplasm of the ascites cell can be divided broadly into two regions—a granular zone of comparatively low ultra-violet absorption, and a peripheral region possessing greater absorption, but with little structure visible by the optical microscope. Absorption measurements at 257 and 275 mμ show that, despite the difference in ribonucleic acid content in these two regions, the protein concentrations are approximately the same.

For comparison with these microspectroscopic results, optical retardations have been measured in the identical regions of the same ascites cells. Total dry mass concentrations, evaluated interferometrically, were found to be in good agreement with the sum of the nucleic acid and protein weights, these being obtained from the ultra-violet results. This indicates that the ultra-violet absorption of the cytoplasm of the fresh ascites cell is not grossly different from that to be expected from its extracted nucleic acid and protein components. It is stressed that the material used in this investigation was in many ways suitable for quantitative absorption measurements and equally consistent results should not be expected from all other types of cells, particularly those measured after fixation rather than in a fresh condition.

Introduction

THE determination of cellular nucleic acid and protein content by a microspectroscopic method involves a number of assumptions and is usually open to many possible errors. It is therefore not suprising that over the past few years, much criticism has been directed towards certain aspects of this technique (for a general critique of microspectroscopic methods, see Pollister, 1955). The recent introduction of the interference microscope into cytological research provides an independent method for checking the validity of certain microspectroscopic results. The interference microscope allows the

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optical retardation produced by a cell to be measured and from this the cellular dry weight can be determined (Barer, 1952; Davies and Wilkins, 1952). This paper describes how a combination of these techniques has been used to investigate the composition of the cytoplasm of fresh ascites tumour cells of mice.

In many ways cells from an ascites tumour are particularly suitable for quantitative microscopic investigation. The tumour cells are suspended and homogeneously distributed in a fluid medium and can therefore be assumed to be under identical nutritional conditions; this results in a remarkably constant nucleic acid content per cell, according to the extensive biochemical measurements of Klein (1950). This finding was supported by the uniform appearance of these cells under the ultra-violet microscope, in contrast to the more variable absorption of cells from corresponding solid tumours. A preparation of fresh ascites cells, either in their own fluid or in physiological saline, can be easily mounted between slide and coverslip. For the measurement of cellular optical densities, the incident illumination can be recorded in the fluid immediately surrounding the cell; any error due to unevenness of illumination over the object field is thus reduced to a minimum.

The importance of using fresh material in an investigation of this kind should perhaps be stressed as even the best fixation methods will introduce changes in the cell; cellular constituents which absorb ultra-violet radiation may be removed, redistributed, or changed in chemical form by the process. Moreover, the precipitation effects normally associated with fixation techniques will tend to increase the amount of light scattered by the material, and the necessary correction is, in general, difficult to evaluate. Recent ultra-violet measurements by Davies (1954) on the same cells, first in the living state and then after fixation, showed considerable differences in the shape of the ultra-violet absorption curves; it is suggested by this author that the derivation of relative amounts of nucleic acid and protein from the absorption spectrum of the living or the fixed cell must be viewed with caution.

Experimental Methods

Material. In this investigation, cells from the Landschütz and Ehrlich ascites tumours have been used; the two tumours are very similar, the former possessing a narrower distribution of chromosome numbers and fewer mitotic abnormalities (Ledoux and Revell, 1955).

In their natural roughly spherical shape, the fresh tumour cells are too refractile for any detailed examination of internal cellular structure, while they absorb the ultra-violet radiation too strongly to be suitable for microspectroscopic measurement. These disadvantages can be overcome, however, by squashing the cells to a thickness between 5 and 10μ. Moreover, the cells apparently suffer little damage under this condition and at room temperature remain unchanged in their morphological and absorption characteristics for periods up to several hours. For measurement, the cells have been suspended either in their own ascitic fluid or in physiological saline.

Ultra-violet microscopy. The ultra-violet system consisted basically of a Cooke, Troughton, and Simms ultra-violet microscope (Taylor, 1953) which has been modified for quantitative absorption measurements by a photographic technique (King and Roe, 1953). Although the absolute accuracy of photographic densitometry is limited to approximately 5%, comparison measurements under similar conditions, and in particular from photomicrographs of the same cell at different wavelengths, can be made to a somewhat higher accuracy.

Measurements have been confined to three wavelengths, 257 mμ and 275 mμ obtained from a cadmium spark source and conveniently located close to the absorption maxima of nucleic acids and proteins respectively, and 313 mμ, obtained from a medium pressure mercury arc and used for scatter measurements. To simplify the experimental procedure, a single monochromat has been used at all three wavelengths (objective N.A. = 1·25, condenser N.A. = 0·55). A quartz monochromat is corrected for one specific wavelength in the ultra-violet, and used away from that wavelength will exhibit some spherical aberration. However, extensive tests on model absorbing particles under the above conditions have shown that this aberration produced no significant error in the optical densities measured (King, 1956).

As some of the densities measured were relatively high (up to 0·7), it has been necessary to apply a correction for glare illumination. Glare is caused by reflection and scattering of the radiation in the image-forming system; it produces a reduction in image contrast, the percentage error in the measured density increasing rapidly at high values (see, for example, Davies and Walker, 1953). The magnitude of this glare illumination has been determined experimentally by measuring the apparent optical density of an opaque carbon particle, under similar optical conditions to those used for the cellular measurements. It was found to be approximately 3% of the incident illumination.

Absorption measurement at a larger number of wavelengths have been made with a Burch reflecting microscope, the specimen being illuminated by the complete radiation from a medium-pressure mercury arc. Dispersion was produced by a quartz spectrograph situated with its entrance slit at the image plane of the microscope (King, 1956). A system of this type has been employed for cellular absorption measurements by Barer, Holiday, and Jope (1950).

Photographic procedure and microphotometry. Ilford N. 40 process plates developed in Kodak D 19b developer have been used, giving a γ-value of 1·4 to 1·5 at 257 mμ. Evenness of development, an essential condition for accurate results, was obtained by moving a perspex blade slightly above the emulsion surface, so ensuring continual replacement of any exhausted developer.

The optical densities of the processed plates were measured with a Hilger non-recording microphotometer, which had been modified first to facilitate the measurement of these densities and secondly for use as a recording instrument. The illumination from this microphotometer is measured by a Baldwin photometer, consisting of a vacuum photocell and a simple d.c. balanced

amplifier. For manual operation, the amplifier output is registered on a micro ammeter, with an approximately linear density scale. An Elliottronic potentiometer recorder, connected across a resistance in the output of the photometer unit, is used for recording, the plate carriage being driven by a synchronous motor. The microphotometer aperture corresponded to a diameter of 0·5 μ at the specimen level.

Specimen irradiation. As in this investigation, fresh rather than fixed material has been used, it is important to know how the cells are affected by the ultra-violet irradiation occurring during photomicrography. It must be pointed out that this irradiation is very considerable (about 10^5 ergs/sq. cm. at 257 mμ; see King and Roe, 1956).

The specimen was searched and focused with a visible phase-contrast system; any ultra-violet irradiation before photomicrography was thus avoided. Phase-contrast examination showed that the cellular structure was not visibly affected by the ultra-violet radiation necessary to obtain the first photomicrograph. Although the ultra-violet absorption of the cell may be altered by this dose, this would seem to be unlikely as further irradiation at 257 mμ, sufficient for at least 10 more photomicrographs, produced no immediate change in cellular absorption.

With the system utilizing a Burch reflecting microscope and ultra-violet spectrograph, the specimen irradiation necessary to record optical densities at the wavelengths emitted by a medium-pressure mercury arc was somewhat higher, but still less than that producing immediate visible changes in the living cell. It must be stressed that with a continuous light source, the resulting specimen irradiation is too large for measurements to be made on living material.

Interference microscopy. For dry mass determinations, the optical retardation due to the cell has been measured with a Baker interference microscope (see Smith, 1955); a shearing-type objective of N.A. 0·7 was used. Köhler illumination was employed, monochromatic light of wavelength 546 mμ being obtained from a 250-watt high-pressure mercury arc with Corning narrow band pass filter (No. 4–102). Measurements were taken with the rotatable analyser, a rotation of $\theta°$ between minimum specimen and minimum background intensities corresponding to $\theta°/180°$ wavelengths of optical path difference between the specimen and surrounding fluid. For some measurements, a half-shade eyepiece was used. Intercalibration of the stages of the interference and U.V. microscopes allowed the same cells to be readily examined on both instruments.

Spectroscopic Analysis

At wavelengths above 250 mμ, cytoplasmic absorption will normally be due to ribonucleic acid and protein and it should be possible to determine the concentrations of these components by an analysis of the type used by Thorell (1947). Basically the method involves the comparison of the cellular optical density at 257 mμ and 275 mμ with extinction values obtained from dilute

bonucleic acid and protein solutions of known concentration. The latter can e referred to as standard absorption curves, for they will form the basis of ny microspectroscopic determinations.

For this analysis, ribonucleic acid has been extracted from the cytoplasm f Landschütz ascites tumour cells by the method of Colter and Brown (1956). 'he absorption curve obtained from the complete ribonucleic acid fraction, recipitated with ethanol, had the following characteristics:

Extinction (257 mμ) of 1 cm of 1% solution = 185, extinction ratio $E_{257}/E_{275} = 1{\cdot}59$ (King and Lawley).

It must be stressed that absorption curves of ribonucleic acids extracted rom different tissues and by different methods vary somewhat both in their naximum extinction (about 257 mμ) and in their extinction ratio E_{257}/E_{275}. 'he former factor will affect the accuracy of cellular ribonucleic acid estima- on, while it will be seen later how the protein determination is controlled y this RNA extinction ratio. Furthermore, it is important to point out that n this analysis, the term 'ribonucleic acid' is used to refer to all the cytoplasmic naterial containing purine and pyrimidine, regardless of its degree of poly- nerization. The implications of this will be discussed later.

Protein absorption at wavelengths above 250 mμ is largely due to its tyro- ine and tryptophane content and is usually estimated from a certain assumed omposition of these aromatic amino-acids (Caspersson and Santesson, 1942; 'horell, 1947). Absorption between 250 and 260 mμ due to phenylalanine and ystine in the protein will be smaller and not sufficient to affect the following nalysis to any significant extent. In certain fibrous proteins, Schauenstein 1950) claims to have detected absorption due to the peptide framework, the bsorption curve possessing a flat contour with a maximum at about 250 mμ. 'he results have been strongly criticized by other workers (see, for example, Beaven and Holiday, 1952; Seeds, 1953), and it appears unlikely that this peptonal' absorption will be of significance in cellular absorption measure- nents. Results of amino-acid analyses from various tissue proteins (Block and Bolling, 1945) have given a tyrosine : tryptophane ratio of about 3 : 1, there eing some variation in the absolute concentrations of these constituent amino- cids. A protein analysis on whole ascites cells gave results similar to those rom tissue proteins; the tyrosine content was approximately 4·5%, trypto- hane not being estimated (Easty). A standard protein absorption curve, ssuming a 4·5% tyrosine and 1·5% tryptophane composition, is shown lotted in fig. 1, curve 1. The tyrosine and tryptophane absorption curves sed are those due to Beaven and Holiday (1952). Ionization of the phenolic roup of tyrosine causes gross changes in the absorption spectrum of this mino-acid between pH 8 and pH 12, and it is therefore essential to use the esults from acid rather than alkaline solution.

From these standard RNA and protein absorption curves, it is then possible o calculate the relationship between the optical density ratio D_{257}/D_{275} nd the ratio of the concentrations of ribonucleic acid and protein (NA/P);

this is expressed by the following equation and is shown plotted in fig. 2 curve 1.

$$X = D_{257}/D_{275} = \frac{185\,\mathrm{NA} + 3{\cdot}9\,\mathrm{P}}{116\,\mathrm{NA} + 7{\cdot}5\,\mathrm{P}}. \qquad (1$$

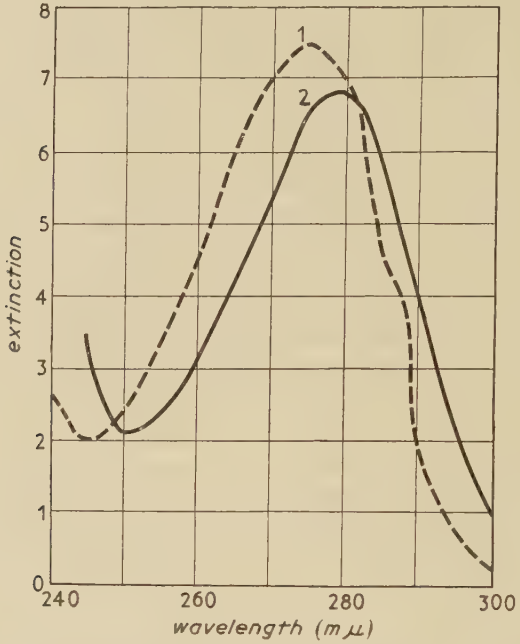


FIG. 1. Absorption curves (1 cm, conc. 1%) of standard protein: 1, derived from $4\frac{1}{2}$% tyrosine, $1\frac{1}{2}$% tryptophane composition; 2, derived from ascitic fluid absorption.

At this point it is relevant to stress that for equation (1) to be valid, it is necessary to assume that the absorption of the nucleoprotein material within the living cell can be obtained by direct addition of that due to its nucleic acid and protein components and is not affected by complex formation. Absorption measurements by Nurnberger (1955) on mixtures of nucleic acid and serum protein solutions gave results 5 to 10% higher than the theoretically additive values. Changes of this amount would have little significance in cellular estimations, but in the living cell, spectral changes due to interaction effects may, of course, be considerably larger.

It will be recalled that the standard protein absorption curve was derived from a certain assumed tyrosine and tryptophane content. A second method has been used to determine this curve; it possesses the advantage that no absolute composition of these amino-acids has to be assumed. This method is based on the absorption characteristics of the peritoneal fluid, in which the living tumour cells are suspended and nourished. The protein, ribonucleic acid, and free nucleotide content of this fluid have been determined by Ledoux and

Revell (1955), the first of these constituents being by far the largest at all stages of the tumour growth (approximately 20 times the sum of the ribonucleic acid and free nucleotide content). Apart from inorganic salts, the concentration of other constituents, e.g. glucose (shown by Klein and Klein, 1956, to be very low), are likely to be small; it would therefore seem reasonable to

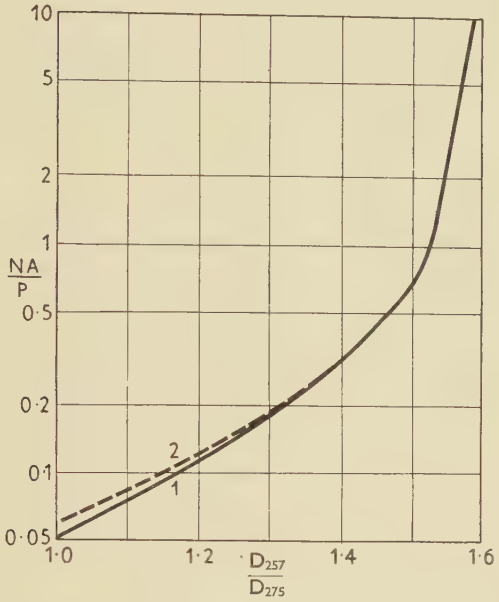


FIG. 2. Relationship between ratio of nucleic acid and protein concentrations and optical density ratio D_{257}/D_{275}.

consider the dry mass of the fluid as being essentially protein, allowing a correction for inorganic salts. The concentration of these salts is taken as 0·8%, approximately that present in plasma. The following analysis is given for one particular ascitic fluid and a comparison of results from various fluids is then described.

Fig. 3 shows the absorption curve of 1 mm thickness of a typical ascitic fluid, obtained as the supernatant after centrifugation of the tumour at 3,000 r.p.m. for 3 min. It is assumed that the optical density D of 1 mm of this fluid is the sum of the densities of its protein and ribonucleic acid components, D_P and D_{NA} respectively. The latter can be related by a constant factor k to the standard extinction E of 1 cm of a RNA solution of concentration 1 g/100 ml, so that

$$D = D_P + kE.$$

Then if D′ and D'' refer to values at 250 mμ and 275 mμ respectively,

$$\frac{D''_P}{D'_P} = \frac{D'' - kE''}{D' - kE'}. \qquad (2)$$

The wavelengths 250 mμ and 275 mμ, near to the minimum and maximum of the ascitic fluid absorption curve, are used to minimize any error due to wavelength shift of the absorption bands (see below).

At this stage it is assumed, as in the previous analysis, that the ratio of tyrosine to tryptophane in the protein is 3 : 1, and thus from fig. 1, curve m

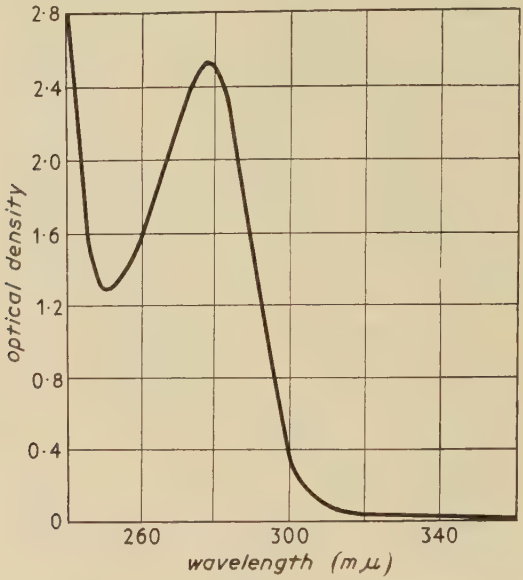


FIG. 3. Absorption curve (1 mm thickness) of typical ascitic fluid.

D''_P/D'_P is equal to 3·1. The factor k can then be evaluated from equation (2), resulting in the following equation, which holds for any given wavelength:

$$D_P = D - 0{\cdot}0033E. \qquad (3)$$

In protein solutions, the following relationship has been shown to exist between the concentration C, expressed in grams of dry material per 100 ml of solution, and the refractive index N of the solution (for general summary, see Barer and Joseph, 1954; Davies and others, 1954):

$$C = \frac{N - N_w}{\alpha}, \qquad (4)$$

where N_w = refractive index of water (i.e. pure solvent) and α = constant, known as specific refractive increment = 0·0018 (approx.) for various proteins and certain other cellular constituents.

Measurement of the fluid refractive index thus enabled the protein concentration to be determined, with allowance for a correction due to the presence of inorganic salts in the fluid. The specific refractive increments of these salts are approximately the same as those of proteins, and in applying this

orrection, a mean value of 0·0018 was therefore used. The ascitic fluid used n this analysis possessed a refractive index of 1·3403, corresponding to a con-entration of dry material of 3·9%, 3·1 g/100 ml being taken as protein. From his information, combined with the absorption of the protein component erived from equation (3) the absorption curve of 1 cm of the standard pro-ein (concentration 1 g/100 ml) was determined and is shown plotted in fig. 1, urve 2. Compared with curve 1 (derived from the constituent tyrosine and ryptophane absorptions), it can be seen that the maximum extinction is ightly lower and is shifted to longer wavelengths by approximately 5 mμ. shift to longer wavelength, which may exceed 3 mμ for the tryptophane ne-structure maximum, has been found in various proteins (Beaven and Holiday, 1952).

Fig. 2, curve 2, shows the relationship between NA/P and X, the optical ensity ratio, obtained from this second standard protein absorption curve. t is interesting to note that repetition of the analysis for a tyrosine : trypto-hane ratio between 2 : 1 and 6 : 1 (rather than the 3 : 1 value assumed) pro-uced no significant change in this final relationship; moreover, it is unlikely hat the ratio of these amino-acids in the cell protein will be outside these mits. Furthermore, results obtained from the fluids of various Landschütz scites tumours showed good agreement in the relationship between NA/P nd X. This held both for tumours examined at random times over a period f more than a year during its propagation and also for tumours of various hysiological ages (except at the very final stages of tumour growth). The use f this second standard protein curve for the estimation of cell content is based n the assumption that the intra- and extra-cellular proteins have similar tyro-ne and tryptophane compositions, although the actual concentrations of ese proteins will, of course, be quite different. The close agreement between sults obtained from various ascitic fluids and from the usually assumed rosine and tryptophane content has prompted the use of this standard ab-rption curve.

Microspectroscopic Results

Cellular morphology. The typical morphology of the interphase ascites cell, uashed to a thickness of approximately 6·5μ, is shown in fig. 4, A. This is an tra-violet photomicrograph taken at 257 mμ and it shows very clearly how e cytoplasm of this cell type can be broadly divided into two regions. One ne, adjacent to the nucleus, contains most of the particulate material of the toplasm and has a comparatively low ultra-violet absorption. The second gion, which is at the periphery of the cell, is far more homogeneous in pearance, little structure being visible by the optical microscope. The sorption in this region is higher, owing to a greater concentration of material ntaining purine and pyrimidine. Although the squashing of the ascites cell phasizes the division of the cytoplasm into two regions, this separation pears to exist in the cell in its natural spherical shape (King and Roe, 1956; s reference contains a fuller description of structure of the ascites cell).

Except at its periphery, the squashed ascites cell can be regarded as a parallel-sided plate of absorbing material of thickness equal to the separation between slide and coverslip. This separation has been determined experimentally by focusing with a high-power phase-contrast system on fine debris attached to the surfaces of the slide and coverslip, great care being taken to ensure that the debris was actually at these surface levels. The true thickness is, of course, equal to the apparent thickness, obtained from the microscope fine motion, multiplied by the ratio of refractive indices of the ascitic fluid and the objective immersion medium. The fine motion of the C.T.S. microscope is calibrated to $0{\cdot}1\,\mu$ and allowed this thickness to be determined accurately, the standard deviation of four thickness measurements being about $0{\cdot}10$ to $0{\cdot}15\,\mu$.

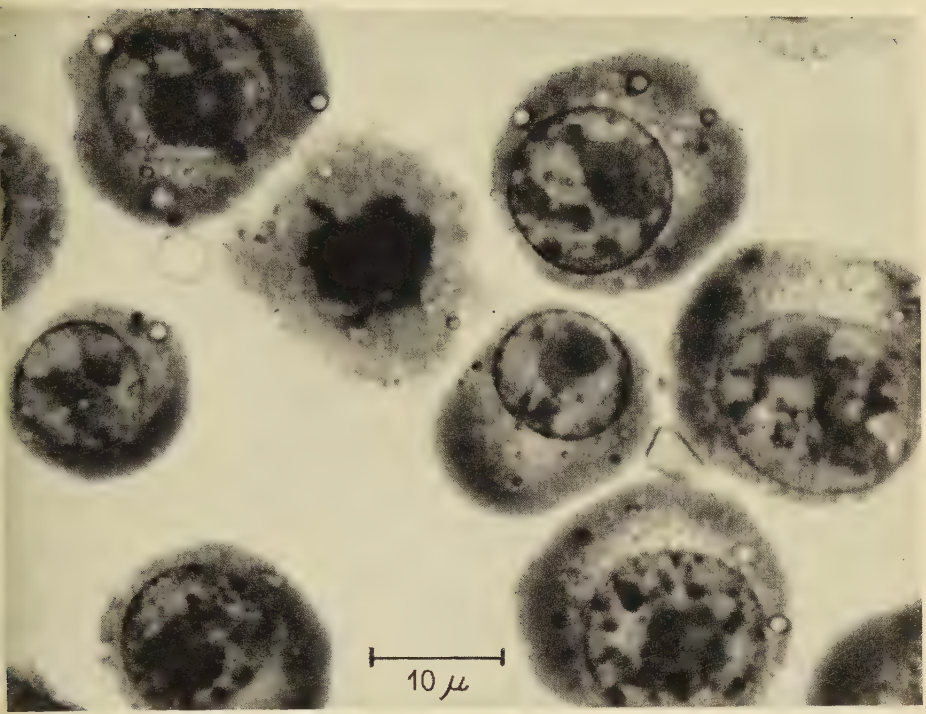
A microphotometer trace ($\lambda = 257$ mμ) across part of a squashed ascites cell is shown in fig. 5, the optical densities being referred to a zero value at *a* in the surrounding fluid. From *a* the density increases to a maximum at *b*, corresponding to the absorption of the homogeneous region of the full thickness between slide and coverslip. The lower optical density of the granular zone of the cytoplasm is shown at *c*; *d* marks the nuclear membrane, the strong absorption of the chromatin being recorded at *e*.

It should be noted that in addition to applying a correction for glare illumination by the method already described, it is necessary to correct for the absorption of the ascitic fluid itself, as the cellular optical densities are measured relatively to this fluid. This correction, which is of particular importance in the determination of the optical density ratio D_{257}/D_{275}, is readily evaluated from the absorption curve of the ascitic fluid.

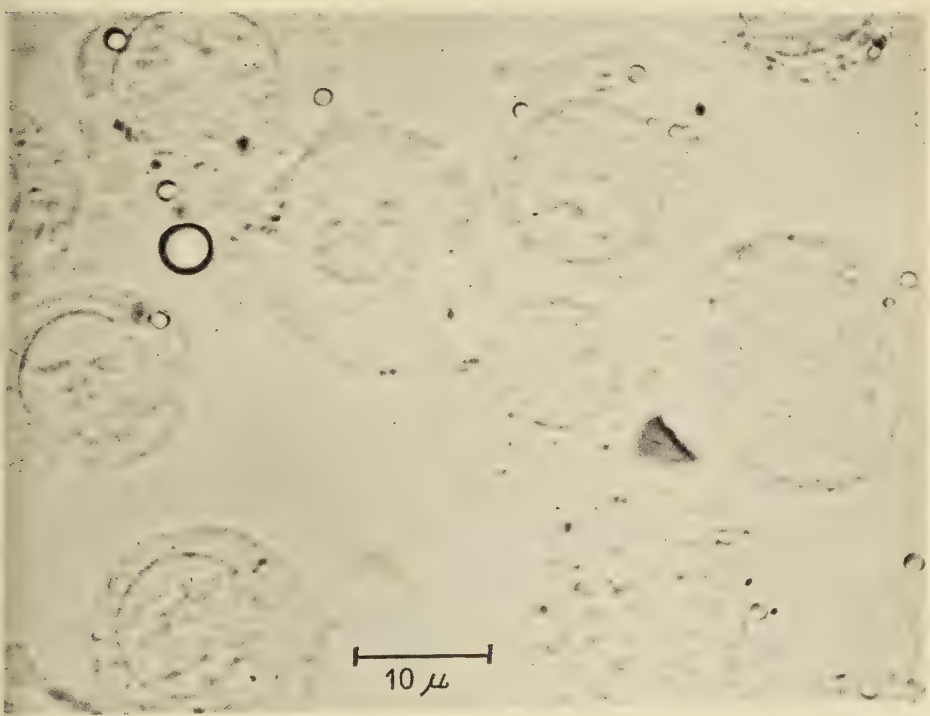
Homogeneous cytoplasmic region. Fig. 6 represents a typical absorption spectrum from the homogeneous region of a fresh, squashed ascites cell, obtained by the Burch microspectroscopic system. The curve possesses the characteristic nucleic acid absorption maximum at about 257 mμ, the cellular absorption falling to extremely low values above 300 mμ. Measurements on a number of cells at wavelengths beyond the absorption bands of nucleic acids and proteins (i.e. at 313 mμ and 334 mμ) gave extremely low optical densities usually not more than 2% of the maximum density at about 257 mμ. This is demonstrated very clearly in fig. 4, A, B, which shows photomicrographs of the same group of cells taken at 257 mμ and 313 mμ respectively. Although scatter will increase at shorter wavelengths, it seems unlikely that in these absorption measurements it will be sufficient to cause appreciable error. In this respect this cytoplasmic region is ideal for quantitative measurements.

Measurements of the optical density (at 257 mμ) per micron thickness have been made on this homogeneous cytoplasmic region in a large number of cells from various Landschütz and Ehrlich ascites tumours. This value clearly depends on the concentration of absorbing material; it should be close

Fig. 4 (plate). Ultra-violet photomicrographs of squashed Landschütz ascites tumour cells at: A, 257 mμ. B, 313 mμ.



A



B

FIG. 4

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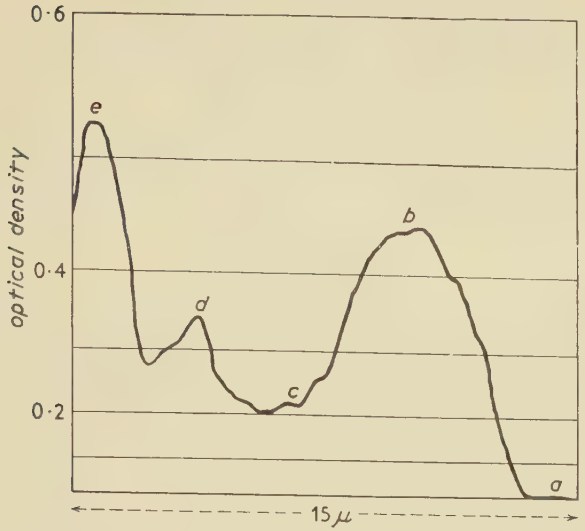

FIG. 5. Microphotometer trace (257 mμ) across part of a squashed ascites cell.

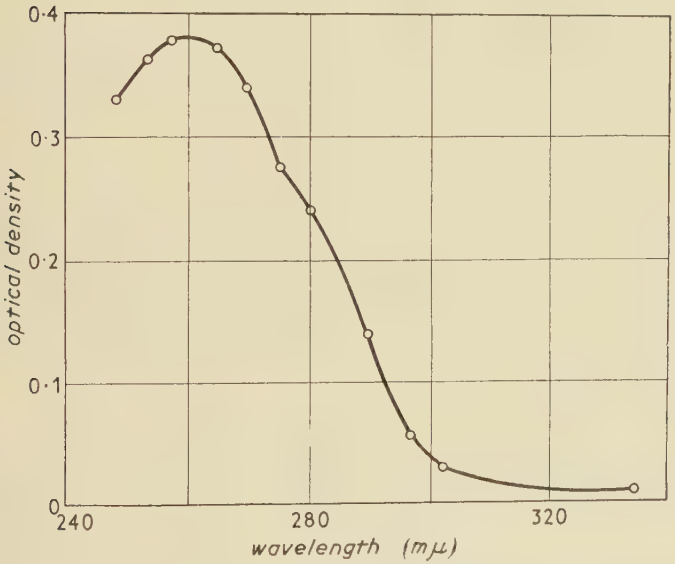

FIG. 6. Typical absorption spectrum from the homogeneous region of ascites cytoplasm.

roportional to the concentration of material containing purine and pyrimi-ine, as the absorption at this wavelength due to protein will be relatively mall (approximately 5%, calculated from the results presented below), and it is nlikely that there will be other material present with any significant absorption

at this wavelength. Assuming the absorption to be due to ribonucleic acid, the mass M per cubic micron of this component will be given by:

$$M = \frac{1}{E}\left(\frac{D}{\mu}\right)10^{-10}\,\text{g},$$

where E is the extinction (at 257 mμ) per centimetre of a RNA solution of concentration 1 g/100 ml.

Therefore $$M = 0{\cdot}54\left(\frac{D}{\mu}\right)10^{-12}\,\text{g}. \qquad (5)$$

It has been found that for interphase cells from a given tumour, the D/μ value was approximately constant, the standard deviation being about 10%. The mean value varied from tumour to tumour, depending on the physiological age of the tumour (i.e. the time from the inoculation of the ascitic fluid into the host animal) and on the characteristics of the cells used in this inoculation. From measurements on 15 Landschütz ascites tumours, a good correlation was obtained between the above (D/μ) value and the ratio (RNA+FN)/DNA, where RNA, FN, and DNA refer to the cellular ribonucleic acid, free nucleotide, and desoxyribonucleic acid contents, these being measured biochemically on the cells in bulk. (The biochemical determinations were made by Dr. Ledoux; for details of methods, see Ledoux and Revell 1955.) On the assumption that the DNA content of the tumour is proportional to the number of cells, a correlation then existed between the cytoplasmic concentration of ribonucleic acid and free nucleotides and the total amount of these constituents per cell.

The value of the optical density ratio D_{257}/D_{275}, measured in this homogeneous cytoplasmic region, has been found to be very constant for cells from a single Landschütz tumour, the standard deviation being approximately 3%. This constancy also held for ratios obtained from various Landschütz tumours, the mean values from 7 different tumours ranging between 1·38 and 1·41. From fig. 2 it can be seen that this corresponds to a nucleic acid to protein content ratio of 0·25 to 0·30. Similar results were obtained from a number of Ehrlich ascites tumours.

Results from those cells used both for microspectroscopic and interferometric measurement are shown in table 1. The ribonucleic acid concentration was calculated from the optical density at 257 mμ, with a correction for protein absorption, while from the ratio D_{257}/D_{275}, the protein concentration has been evaluated.

Granular cytoplasmic region. Measurements have been made on the granular region of the cytoplasm, densities being recorded in the zone where absorption was a minimum and approximately constant (i.e. comparatively free from light-scattering particles in or slightly out of focus). In this region, the optical density ratio D_{257}/D_{275} was about 1·20 to 1·25, corresponding to a nucleic acid to protein ratio of approximately 0·13. Light-scatter from this region was somewhat higher than that from the homogeneous zone, the optical density

at 313 mμ being on average about 5% of the value at 257 mμ. However, calculations based on the assumptions that the scatter is either independent of the wavelength or varies inversely as the fourth power of the wavelength showed that even in these extreme cases, an optical density ratio of 1·25 would not be significantly affected by scatter of this magnitude. Possible anomolous dispersion in the region of an absorption band could, of course, result in larger scatter effects (Scott, 1952).

TABLE I

Ultra-violet absorption and interferometric measurements of the cytoplasm of Landschütz ascites tumour cells

Tumour:	*Homogeneous region*				*Granular region*	
	A	*B*	*C*	*D*	*C*	*D*
Physiological age (days)	6	7	9	7	9	7
No. of cells measured	8	6	11	7	9	7
Mean D/μ (257 mμ)	0·090	0·064	0·093	0·084	0·045	0·044
Mean M (10^{-12} g) (RNA mass per cu. μ)	0·046	0·033	0·047	0·043	0·020	0·019
D_{257}/D_{275}	1·38	1·39	1·41	1·38	1·23	1·24
RNA/P	0·26	0·27	0·31	0·26	0·13	0·135
Mean P (10^{-12} g) (protein mass per cu. μ)	0·177	0·120	0·152	0·155	0·154	0·141
M+P (10^{-12} g)	0·22	0·155	0·20	0·20	0·175	0·16
Mean M (10^{-12} g) (total dry mass per cu. μ)	0·18	0·14	0·18	0·23	0·22	0·27

Results from the granular region are also shown in table I; ribonucleic acid concentrations were determined from equation (5), a correction being applied to allow for the effects of protein absorption and light-scatter in this region, the latter was assumed to be independent of wavelength.

Interferometric Results

The use of the interference microscope to determine the dry mass of a biological structure is based on equation (4), relating the refractive index of a solution to the concentration of dissolved material. The equation has been found to be valid up to concentrations as high as those occurring in the living cell; the value of the specific refractive increment is approximately 0·0018 for most cellular constituents, being somewhat lower for carbohydrates and some inorganic salts (see, for example, Barer and Joseph, 1954).

It can then be shown that the dry mass $\bar{M}$ per cubic micron is given by:

$$\bar{M} = \frac{1}{\chi t}[\phi+(N_m-N_w)t]10^{-12}\,\text{g},$$

where N_m and N_w are the refractive indices of the medium surrounding the

cell, and of water respectively; t = thickness of structure in μ; ϕ = optical retardation produced by cell in μ, measured relative to surrounding fluid; $\chi = 100\,\alpha$, where α = specific refractive increment $\simeq 0{\cdot}0018$.

For these interferometric measurements, it was essential that the microscope field contained only a small number of cells, so that the reference beam in this shearing-type system could be arranged to traverse an area free from cells. This was achieved by diluting a small volume of the tumour with a larger quantity of the cell-free ascitic fluid, obtained as the supernatant layer after centrifugation. Retardations were determined relatively to the ascitic fluid immediately surrounding the cells, the refractive index of this fluid being measured with an Abbe refractometer.

For comparison with the microspectroscopic results, optical retardations were measured in the identical regions of the same ascites cells, the two series of measurements being made in rapid succession to minimize any error due to possible cellular changes. Total dry mass concentrations, evaluated from the interferometric measurements, are also included in table I.

It should perhaps be stressed that although these comparison measurements were made only on about 30 cells from 4 tumours, the results tabulated are characteristic of the much larger number of cells subjected to either microspectroscopic or interferometric analysis. Thus, the interference results from the homogeneous and granular regions of the same cells (i.e. from tumours *C* and *D*) indicate that the dry mass is approximately 15 or 20% greater in the latter region than in the homogeneous zone; measurements on cells from other ascites tumours confirmed this finding.

Discussion

This investigation has illustrated many of the difficulties inherent in the determination of cellular content by a microspectroscopic technique. It has been shown that, even if the absorption curve of part of a cell could be measured accurately, the determination of nucleic acid and protein concentrations from it is still open to error due to the uncertainty of the necessary 'standard' absorption curves. In practice, these cellular absorption measurements are usually limited in accuracy, the very structure of the protoplasm causing appreciable light scatter. Moreover, unless adequate precautions are taken, further errors can easily arise from the microscope and its associated optical system (King and Roe, 1953).

On the other hand, the use of the interference microscope for total dry mass determinations is generally considered to be based on sounder principles. With this technique, many of the difficulties associated with microspectroscopy are either avoided completely or their effect considerably lessened. It is consequently of interest that results obtained by these two independent methods are in good agreement (table I). By slight modification of the standard absorption curves, the microspectroscopic results from the homogeneous cytoplasmic region could be brought into even closer agreement with the dry mass determinations. Results from the granular zone would, however, then show a larger

difference, but this could be due to the presence of material other than nucleoprotein, of lipid type for example, with no significant absorption in the ultraviolet region employed. The staining methods used by Love and others (1956) on fixed ascites smears have shown quite clearly the sudanophil nature of the granular cytoplasmic region.

Moreover, it is important to stress that the cytoplasmic material containing purine or pyrimidine is unlikely to be all in a highly polymerized form, but will exist at various molecular weights, down, perhaps, to the level of single nucleotides. Now, it is probable that the absorption will be dependent on this degree of polymerization (Magasanik and Chargaff, 1951) and although this effect may only produce a small error in the determination of material containing purine and pyrimidine, a change in shape of the absorption curve may result in much larger errors in the estimation of protein. The latter will inevitably be severely limited in accuracy by the relatively low ultra-violet absorption of proteins. Obviously the combination of ultra-violet and interference microscopy will allow a more comprehensive measure of cellular constituents than either method can achieve alone.

It can, however, be concluded that the ultra-violet absorption of the nucleoprotein complex in the cytoplasm of the living ascites cell is not grossly different from that to be expected from its extracted nucleic acid and protein components. Moreover, from biochemical measurements on ascites tumour cells (Ledoux and Revell, 1955) a (RNA+FN)/PROTEIN ratio of between 0·1 and 0·2 was obtained, the exact value depending on the physiological age of the tumour. As these results referred to the whole cell, no accurate comparison can be made with the microspectroscopic determinations, but it can be seen that the ratios obtained by both methods were about the same.

The constancy of the optical density ratio D_{257}/D_{275} in the homogeneous cytoplasmic region of cells from various Landschütz ascites tumours is also of interest. It points to a roughly stoichiometric relationship between the ribonucleic acid and protein in this region of the cytoplasm; this constancy was found in tumours with physiological ages ranging from one to 9 days. The spread of values obtained from different cells of a single tumour may indicate true variations in the nucleic acid and protein composition, but, owing to the limited experimental accuracy of the technique, may not be significant. However, measurements on over 150 cells have not produced an optical density ratio D_{257}/D_{275} greater in value than that found in isolated ribonucleic acid. This is in contrast to the results of Davies (1954), on the nuclei of living chick fibroblasts, where the measured density ratio was sometimes higher than that for isolated desoxyribonucleic acid, a finding that is difficult to interpret.

It must be stressed that the material used in this investigation was in many ways suitable for quantitative absorption measurements and equally consistent results should not be expected from all other types of cells. In particular, cellular material possessing a less homogeneous distribution of its absorbing contents and capable of producing greater light scatter will be less suitable for microspectroscopic study. Moreover, the measurements have been confined

to small areas of typical cytoplasmic regions, where the absorption is roughly constant; integration of measurements to determine the total content of material that absorbs ultra-violet light usually introduces considerable technical difficulties.

Finally it must be noted that these results were obtained on cells in the fresh state. Preliminary absorption measurements on fixed ascites cells have given more variable results, often significantly different from those obtained with living cells. Rather similar differences between fresh and fixed cells have been reported by Davies (1954), and the validity of results from fixed material appears, at present, to be open to doubt.

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An Examination of the Cuticles of two Scorpions, *Pandinus imperator* and *Scorpiops hardwickii*

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Summary

The cuticles of *Pandinus imperator* and *Scorpiops hardwickii* have been examined histologically and histochemically. They consist of a two-layered epicuticle, a hyaline exocuticle, a quinone-tanned exocuticle, an impregnated but untanned exocuticle, and on the inner surface an unmodified procuticle. The hyaline exocuticle is, however, single in *Scorpiops* and double in *Pandinus*. With the exception of the hyaline exocuticle the cuticles therefore conform to the basic arthropod pattern.

The quinone-tanned exocuticle closely corresponds with that of insects but the hyaline exocuticle seems to have no counterpart in other arthropods. The sulphur content of the cuticle has been determined and it is probable that sulphur is present only in the hyaline exocuticle.

The statement by Krishnan that chitin is present in the epicuticle is critically examined and a possible explanation of the differences which have been reported between the cuticles of different scorpions is offered.

Introduction

THE outermost layers of the arthropod cuticle, collectively termed the epicuticle, have until recently been regarded as not containing chitin (Richards, 1951). In fact, the definition of the epicuticle has been an outer layer devoid of chitin. It was, therefore, surprising when in 1955 Krishnan, Ramachandran, and Santaman claimed that the epicuticle of the scorpion, *Palamneus swammerdami*, does contain chitin. This report, based on X-ray diffraction studies and chromatographic examination, is the first suggestion that chitin may be present in an arthropod epicuticle. Since it is in direct opposition to generally accepted views it has been thought desirable in the present work to examine in some detail the cuticles of two species of scorpions in order to determine whether the findings of Krishnan and his colleagues for *Palamneus* are also true for *Pandinus* and *Scorpiops*.

The cuticles of one adult male *Pandinus imperator* (Koch) and three young of this species at various stages of development, and of one adult specimen of *Scorpiops hardwickii* (Gervais), have been critically examined. All the specimens of *Pandinus* were obtained shortly after death; and as examination of the epidermis and other tissues showed that no drastic post-mortem changes had taken place, it was assumed that the much more durable cuticle was suitable for examination and that the results obtained would be valid. The specimen of *Scorpiops* had been fixed in formaldehyde solution before coming into my possession. Sections of paraffin-embedded or frozen cuticles were stained either with Masson's trichrome or Mallory's triple stain. Both gave good

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results. Histochemical tests were applied to both paraffin and frozen sections. Since they showed no great difference it was assumed that no great amount of cuticular material was lost during the process of paraffin embedding.

It will be convenient first to describe the cuticle of *Pandinus* and then to discuss the differences between it and *Scorpiops*.

The Cuticle of *Pandinus*

The sclerite cuticle (fig. 1). The cuticle of the adult *Pandinus* is very dark brown; this is at least partly due to the presence of melanin in the underlying

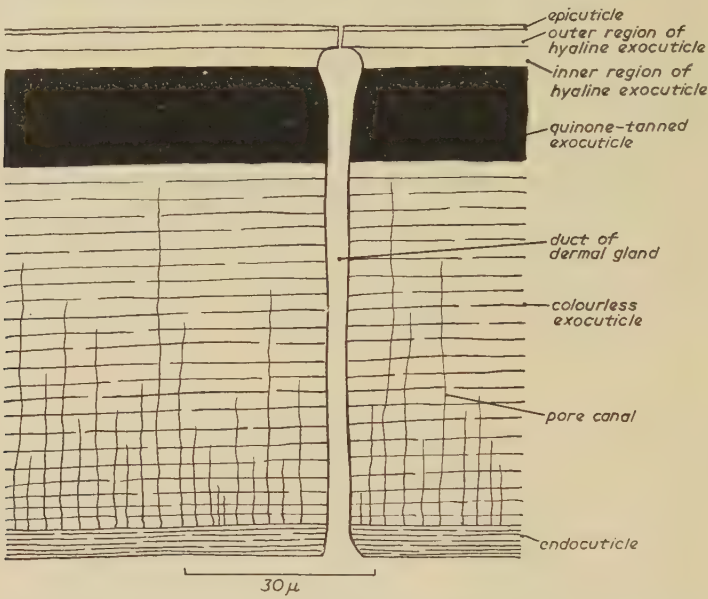

Fig. 1. Transverse section of a fully hardened dorsal abdominal sclerite of *Pandinus imperator*.

epidermis. Unstained sections of a dorsal abdominal sclerite show that the cuticle is about 120 μ thick and is made up of three distinct layers. The main bulk of the cuticle is about 95 μ thick and is conspicuously laminated and colourless. Immediately above it lies a dark brown layer about 15 μ thick and above this is an outer, colourless layer about 10 μ thick. The initial impression given by these three layers is that they constitute the endocuticle, tanned exocuticle, and epicuticle of a typical arthropod, but examination of stained sections does not support this view, at least without modifications. Staining with Mallory's triple stain reveals that the cuticle is further subdivided. It shows that the outermost layer is a blue-staining membrane which is too thin to measure. It can only be discerned in sections that are truly vertical to the surface of the cuticle. Beneath this layer is a red-staining layer about 2 μ thick. The main bulk of the outer colourless layer is completely refractory to staining. The remainder of the cuticle appears to be similar to that of insects. The

dark brown, quinone-tanned exocuticle does not stain but the underlying colourless portion of the cuticle stains differentially; its outer region becomes red and the inner blue with Mallory's triple stain. This condition recalls that seen in, for example, the cockroach *Periplaneta americana* (Dennell and Malek, 1955). The cuticle internal to the tanned exocuticle is traversed by pore canals which take up acid fuchsin.

Not all these layers were described by Krishnan (1953) after Mallory staining. He mentioned the thin blue-staining surface layer but states that the whole of the colourless region outside the tanned exocuticle is refractory to staining: he therefore does not record the presence of a superficial red-staining zone. From his description the remainder of the cuticle of *Palamneus* conforms to that given here for *Pandinus*. In view of the description given by Krishnan it is not surprising that he interpreted the outermost thin blue-staining layer as corresponding to the paraffin layer of the epicuticle of insects. The layer within, refractory to staining, he regarded as representing the cuticulin layer overlying, as is to be expected from a comparison with insects, a tanned exocuticle.

On the other hand, the cuticle of *Pandinus* may be regarded as comprising an outer paraffin layer, a cuticulin layer staining red as often seen in insects especially during development, and intervening between this layer and the tanned exocuticle a colourless refractory layer which seems to have no counterpart in insects. To anticipate the final conclusions of this paper, this layer is referred to as the hyaline exocuticle (fig. 1). On this interpretation the refractory layer of *Pandinus* is internal to the epicuticle whereas in *Palamneus* it seemed to form the main bulk of the epicuticle. The implications of the different staining reactions of the outer zones of the two cuticles, particularly with regard to Krishnan's report of a chitinous epicuticle, will be discussed later.

Just as in insects, the sclerite is bleached by diaphanol; this suggests that quinone-tanning alone is responsible for the colour and hardness of the exocuticle. After this treatment the exocuticle stains with the aniline blue of Mallory's triple stain and closely resembles the unmodified procuticle beneath it. Unlike the arthrodial membrane cuticle to be described below, the sclerite cuticle carries the ducts of dermal glands. The ducts are about 6 μ in diameter and are swollen distally in bulb-like form. At first sight they appear to end blindly about midway through the hyaline exocuticle, but closer examination shows that they continue to the surface of the cuticle, where they open in shallow depressions as very fine tubes (fig. 1). Shrivastava (1954) has described similar ducts in *Buthus tamulus gangeticus* and *Palamneus bengalensis*. Krishnan (1953) on the other hand states that the ducts in *P. swammerdami* end in the layer which he terms the inner epicuticle and which is referred to here as the hyaline exocuticle. No contents of the ducts were observed in either *Scorpiops* or *Pandinus* in the present work but Shrivastava (1954) states that cytoplasm is present in the ducts of *Buthus* and *Palamneus*.

The arthrodial membrane cuticle. Sections of arthrodial membrane (fig. 2)

show 5 cuticular layers to be present. A paraffin layer and cuticulin layer constitute the epicuticle and beneath these a non-staining layer is continuous with the thicker hyaline exocuticle of the sclerite. Unlike the hyaline exocuticle it does not show division into two subsidiary layers. Since it is continuous with the hyaline exocuticle of the sclerite and apparently similar in properties, this colourless layer must also be called the hyaline exocuticle, in spite of the fact

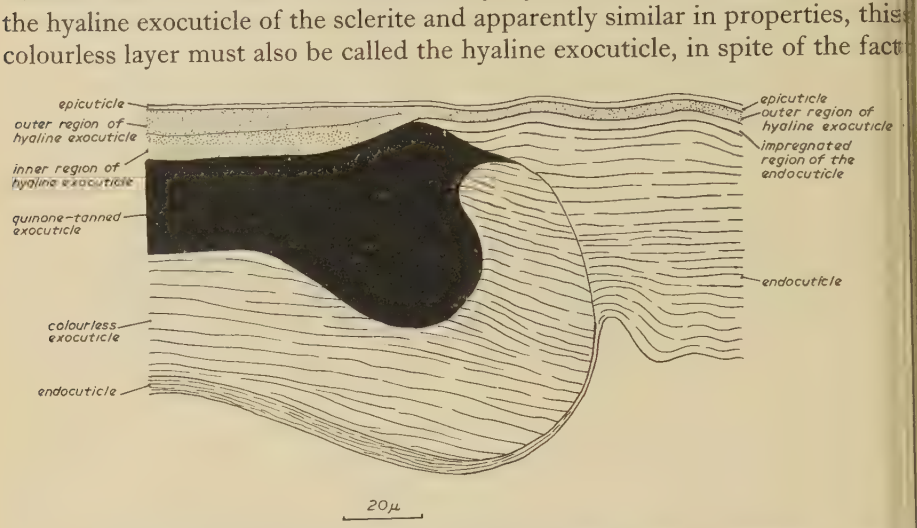

FIG. 2. Transverse section of the cuticle of a fully hardened adult *Pandinus imperator*, to show the transition from sclerite to arthrodial membrane.

that arthrodial membranes are commonly regarded as being devoid of an exocuticle. Beneath the hyaline exocuticle a narrow zone continuous with tanned exocuticle of the sclerite stains red with acid fuchsin. In this it resembles the impregnated but untanned exocuticle of some insects, e.g. *Periplaneta americana* (Dennell and Malek, 1955), and is probably similar in composition. The innermost layer of the arthrodial membrane consists of undifferentiated procuticle which stains blue with Mallory and green with Masson's trichrome stain. Its continuity with the endocuticle is difficult to demonstrate. Intervening between the sclerite and the arthrodial membrane is a zone of thicker cuticle (fig. 2). The increased thickness is due to the expansion of tanned and colourless exocuticles. In contrast the endocuticle of this region appears to be absent, but since an endocuticle appears to be a constant feature of all areas of an arthropod cuticle it must be presumed that an endocuticular layer is present here also but is thin and difficult to detect. The endocuticle has therefore been represented in fig. 2.

The cuticle of young Pandinus. The cuticle of a newly born *Pandinus* is composed of three layers only. A cuticulin layer surmounted by a paraffin layer forms the epicuticle. The remainder of the cuticle consists of undifferentiated procuticle (fig. 3, A). Fig. 3, B shows a small portion of the outer part of the cuticle greatly magnified to show the paraffin and cuticulin layers. At this stage no sign is seen of the hyaline exocuticle. After the first moult the young assumes a light brown colour and examination of the cuticle shortly

after the moult has taken place shows that changes have occurred. The hyaline exocuticle is now present as a pale yellow layer which is subdivided as in the adult into two distinct layers. The inner of these two layers is traversed

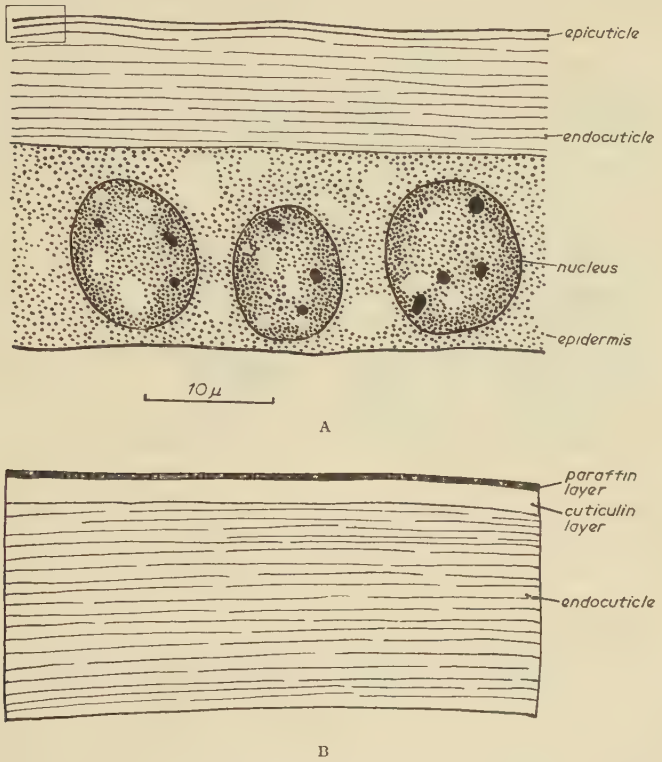

FIG. 3. A, transverse section of the cuticle of a newly born young of *Pandinus imperator*. B, greatly magnified view of the region enclosed by the rectangle in A (not to scale).

by groups of pore canals which take up the acid fuchsin of Mallory (fig. 4). The pore canals cannot be traced into the endocuticle and it can be only assumed that they are present and stain the same colour as the endocuticle. Laminae are present throughout the endocuticle and are regular in appearance. Dermal gland ducts are present and are more closely spaced than in the adult. They arise from enormous gland-cells situated between the epidermal cells. The ducts have the general form of those of the adult cuticle (fig. 1).

The chemical composition of the cuticle. The various histochemical reactions of the fully hardened cuticle are summarized in table 1. The positive chitosan reaction (treatment with caustic potash followed by the application of sulphuric acid and iodine) indicates that the chitin is the main constituent of the cuticle beneath the hyaline exocuticle. The hyaline exocuticle did not give a positive reaction.

After treatment with potash all the region reacting with the iodine and sulphuric acid dissolved rapidly in 3% acetic acid; this further indicates the

presence of chitin. The hyaline exocuticle did not dissolve. Sections of cuticles after treatment with potash tended to split at the margin of the quinone-tanned and hyaline exocuticle. The hyaline exocuticle does not become reactive towards staining and chemical tests after potash treatment.

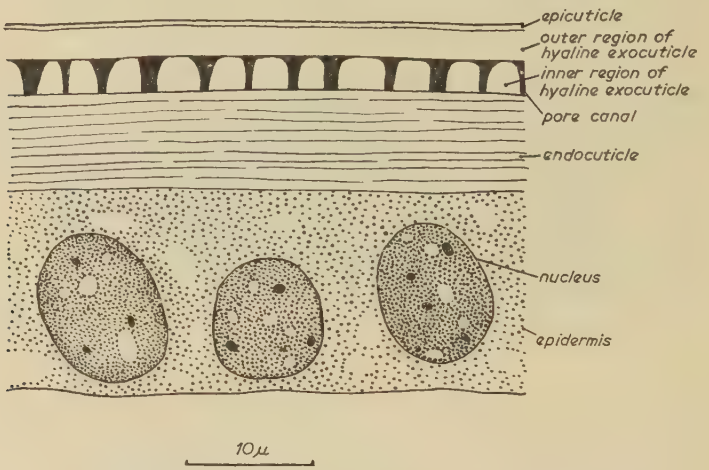

FIG. 4. Transverse section of the cuticle of a young *Pandinus imperator* shortly after the first moult.

TABLE I

Name of test	*Paraffin layer*	*Cuticulin layer*	*Hyaline exocuticle*	*Quinone tanned exocuticle*	*Colourless exocuticle*	*Endocuticle*
Sudan black B	+++	—	— ++	+ -	?	?
Xanthoproteic	—	++	— —	+++	++	—
Millon's	—	+++	— —	+++	+	—
Argentaffin	—	++	— —	+++	+	—
Biuret	—	+	— —	++	+	—
Mallory staining before diaphanol	blue	red	— —	—	red	blue
Mallory after diaphanol	blue	blue	— —	dark blue	light blue	light blue
Masson staining	black	blue	— —	—	red	green
Chitosan	—	—	— —	+++	+++	++
Solubility in conc. nitric acid	on boiling	on heating	on boiling	on warming	in the cold	
Mörner's	—	—	— —	—	—	—

All these observations are in agreement with those of Krishnan (1953) for the cuticle of *Palamneus swammerdami*.

Positive Millon and xanthoproteic reactions were given by the cuticulin layer and quinone-tanned exocuticle. The reaction given by the colourless exocuticle was more feeble and no reaction was detected in the endocuticle. With Mörner's reagent no reaction was given with any part of the cuticle. This is in agreement with the observations of Krishnan (1953), who did not find tyrosine to be present in the cuticle of *Palamneus*. The argentaffin

reaction was strongest in the quinone-tanned exocuticle; it was probably due to the presence here of quinones. A less strong reaction was given by the cuticulin layer and only a very pale brown colour was seen in the colourless exocuticle. The endocuticle did not react.

The staining reactions of the cuticle before and after treatment with diaphanol are interesting. Before treatment the quinone-tanned exocuticle is not stained with any of the components of Mallory's triple stain but the colourless exocuticle and endocuticle stain red and blue respectively. After two weeks' treatment with diaphanol the cuticle was bleached and the staining reactions modified. The bleached tanned exocuticle stained with aniline blue and both the colourless exocuticle and endocuticle stained light blue. This recalls the condition seen in the cockroach, *Periplaneta americana*, where it was found by Dennell and Malek (1956) that treatment of the adult cuticle or any hardened cuticle caused it to stain like the untanned cuticle. After more prolonged treatment with diaphanol the whole region of the cuticle internal to the hyaline exocuticle, instead of staining a definite pale blue, becomes a dull blue-gray. This may be related to the fact that the cuticle now consists of purified chitin. The hyaline exocuticle is not affected in any way by this prolonged treatment and it can therefore only be assumed that quinone tanning is not an important feature of this region of the cuticle. In cold strong nitric acid most of the cuticle dissolves fairly readily but the epicuticle and hyaline exocuticle do not dissolve. With hot or boiling concentrated nitric acid the epicuticle and hyaline exocuticle dissolve slowly. These outer layers do not dissolve in 6N hydrochloric acid at 100° C. even if the treatment is prolonged for 14 days. It was difficult to identify the cuticulin layer after this treatment, but the paraffin layer is still present and stains with aniline blue. These observations and similar ones of Krishnan (1953) seem to be consistent with the view that the stability of the hyaline exocuticle is attributable to sulphur bonding. Using both the alkaline lead acetate and nitroprusside reagents, Krishnan (1953) has been able to show the presence of sulphur in the inner epicuticle (hyaline exocuticle) of *Palamneus*. In the present work, however, neither of these tests gave positive results. Pearse (1953) records that the lead acetate reaction is not given by tissues fixed in either formalin or alcohol. Pearse also found that the colour given by the nitroprusside reagent on tissues fixed in formalin or alcohol was invisible in sections. In the light of these observations the lack of positive results cannot be taken as indicating the absence of sulphur. Chromatographic analysis of the cuticle does not reveal cystine or other amino-acids containing sulphur. If the stability of the hyaline exocuticle is due to sulphur bonding, the apparent absence of sulphur is easily explicable. Krishnan (1953) found that treatment with alkaline sodium sulphide, a reagent said to rupture sulphur bonding, caused the inner epicuticle (hyaline exocuticle) to become reactive to stains. This observation would seem to point to the presence of sulphur, but similar reactions were not given by the cuticle of *Pandinus*.

In view of the failure to detect sulphur by histochemical means in the

cuticle of *Pandinus*, it was decided to carry out a gravimetric analysis of the cuticle according to the method given by Hawk and others (1954). Pieces of cuticle were taken from various regions of the body and dried to constant weight in hot air. The cuticle was then oxidized by the application of concentrated nitric acid and hydrogen peroxide, to which was later added bromine. This treatment converts the sulphur to sulphate, which is estimated as the barium salt. A control experiment was carried out and the difference in sulphate determined. By this method it was found that the total sulphur content of the cuticle was about 4·6%. This appears to be the only information available on the sulphur content of scorpion cuticles. Lafon (1943) records that in *Limulus* the sulphur content is 2·85%. By comparison with *Pandinus* the sulphur-containing region of the cuticle of *Limulus* is much thinner than that of *Pandinus* and is therefore possible that this alone may account for the difference between these figures.

Intense colouring with Sudan black B is confined to the paraffin layer of the epicuticle, which shows as a separate black layer. The inner region of the hyaline exocuticle is coloured to a certain extent as is the quinone-tanned exocuticle. The colourless exocuticle and endocuticle take up very little colour and it is doubtful whether this is significant in these two layers.

The results recorded in this section indicate that except for the presence of a hyaline exocuticle, which presumably contains sulphur, the cuticle of *Pandinus* bears a strong resemblance to the cuticle of insects. It contains chitin and protein, and a quinone-tanned exocuticle is present. The thin surface layer of the epicuticle, which colours strongly with Sudan black B, appears to correspond to the paraffin layer of the insect cuticle.

The Cuticle of *Scorpiops*

Only one adult specimen of *Scorpiops* was available and although this was fixed in formaldehyde solution, sufficient evidence has been obtained to indicate that the cuticle is very similar to that of *Pandinus*. It appears black, but as the tanned exocuticle forms only a small part of the cuticle it is probable that much of the colour of the animal is due to the presence of the considerable amount of melanin or other dark pigment in the epidermal cells. The cuticle of the dorsal abdominal sclerites is about 45 μ thick. The cuticle differs only slightly from that of *Pandinus* and the differences lie in the thickness of the various layers.

The quinone-tanned exocuticle is very thin in *Scorpiops*, seldom exceeding 8 μ in thickness. It is, however, very much darker than that of *Pandinus* and it is possible that this is due to more intensive polymerization. The colourless exocuticle which stains red with Mallory is also thin and never attains a thickness of more than 6 μ. The endocuticle on the other hand constitutes about half the total thickness of the cuticle in this animal, while in *Pandinus* the endocuticle accounts for only a twelfth of the total. The epicuticle of *Scorpiops* is composed of a paraffin layer surmounting a thicker cuticulin layer, these layers staining blue and red respectively with Mallory. Some

difficulty is experienced in interpreting the outer layers of the cuticle, as the surface is uneven and has rounded projections irregularly distributed over its surface. A hyaline exocuticle of *Pandinus* is also present in *Scorpiops* but is composed of only one layer as compared with two in *Pandinus*. The same histochemical reactions are given by the various regions of the cuticle of *Scorpiops* as in *Pandinus* and it seems that the only differences existing between the two cuticles are those of relative thicknesses of the constituent layers.

Discussion

Examination of the cuticles of the scorpions *Pandinus imperator* and *Scorpiops hardwickii* indicates that they conform to the basic pattern of arthropods generally, consisting of a non-chitinous epicuticle and a chitinous procuticle. They do, however, differ to some extent from those of insects and Crustacea in the organization and modification of the procuticle. It is presumed from staining reactions and histochemical tests that the endocuticle, colourless exocuticle, and quinone-tanned exocuticle of the scorpions are of similar constitution to their counterparts in insects. Reactions with diaphanol suggest that sulphur bonding is not prominent in these inner layers. The chemically inert, hyaline exocuticle is not known in insects. This region is divided into two layers in *Pandinus* but no such division is seen in *Scorpiops*, even though the hyaline exocuticle in this animal is quite thick. The significance of the subdivision in *Pandinus* is not clear and the complete lack of reactions of both layers makes it impossible to state whether there is any chemical difference between them.

The epicuticle in both *Pandinus* and *Scorpiops* is two-layered, consisting of a cuticulin layer surmounted by a paraffin layer. The cuticulin layer is proteinaceous as indicated by positive Millon and xanthoproteic reactions. It also stains red with Mallory. The paraffin layer is much thinner than the cuticulin layer and colours with Sudan black B as well as staining blue with Mallory. From these reactions the epicuticle of these scorpions appears to be similar to that of insects.

This interpretation of the cuticle of *Pandinus* and *Scorpiops* is not in full agreement with that of either Krishnan (1953) or Shrivastava (1954). Shrivastava (1954) stated that in the cuticles of *Buthus tamulus gangeticus* and *Palamneus bengalensis* a paraffin layer is not present in the epicuticle. The colourless layer outside the quinone-tanned exocuticle, however, is double-layered as in *Pandinus*. The outer of these layers interpreted as the outer epicuticle is a dried varnish-like layer containing bound lipids, while the inner layer interpreted as the inner epicuticle is less resistant and gives a strong positive reaction with Sudan IV. Krishnan (1953) found that in *Palamneus swammerdami* a very thin colourless layer surmounts the quinone-tanned exocuticle and this is bounded externally by a paraffin layer which stains blue with Mallory as in *Pandinus* and *Scorpiops*. He interpreted this colourless layer as the inner epicuticle. It is, therefore, clear that these authors did not observe, in the scorpions they studied, the cuticulin layer which has been

recognized in *Scorpiops* and *Pandinus*. For this reason both authors refer t the equivalent of the hyaline exocuticle of *Pandinus* and *Scorpiops* as the inne epicuticle or epicuticle.

It is possible that the conflict of the views of Krishnan and Shrivastav with those recorded here may be resolved in the following manner. Krishnan' statement that the epicuticle of *Palamneus* contains chitin presents no diffi culty if it should prove that this layer is truly a hyaline exocuticle and that two-layered epicuticle does indeed exist outside it. It may well be that i *Palamneus* the cuticulin layer is very thin and not easily recognized. It is to b noted that in *Palamneus swammerdami* the hyaline exocuticle is not subdivided whereas in *P. bengalensis* and *Buthus tamulus gangeticus* it forms two layers. I the epicuticle of these scorpions is not readily discernible then it was natura for Shrivastava (1954) to interpret the layers as inner and outer layers of th epicuticle. A study of the development of scorpion cuticles would be of grea value in finally establishing the homology of the layers. It might also be pos sible by this means to obtain more information on the chemical composition of the hyaline exocuticle.

I am grateful to Professor H. Graham Cannon, F.R.S., in whose Depart ment this work was carried out, for giving me the specimens of *Pandinu imperator* and to Dr. Belfield for their identification. I wish to than Mr. M. Saleem for the gift of the *Scorpiops hardwickii* and Mr. E. Browning of the British Museum for its identification.

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The Prothoracic Glands of some Coleopteran Larvae

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Summary

The paired prothoracic glands of the larvae of 15 species of Coleoptera have been described. Each is a syncytial or cellular band attached at its ends to the dorsal and ventral tracheal trunks running from the prothoracic spiracles to the head, or the branches of these trunks; the gland may be spread over one or both of the tracheal trunks to a varying degree, shows no nuclear divisions during postembryonic development, and has no nerve-supply. There seems to be a tendency for the glands to be more anteriorly placed in the primitive groups and more posteriorly placed in the specialized ones. No relationship seems to exist between the morphological and histological nature of the gland and the taxonomic position of the insect, nor is there any proportionality between the size of the gland or its nuclei and the size of the insect. But there seems to be a direct relation between the volume of the whole gland and its total nuclear volume, and an inverse relation between the number of nuclei in the gland and the size of the individual nuclei.

Introduction

EVER since Fukuda (1940, 1941) demonstrated that the prothoracic glands of the silkworm, *Bombyx mori*, play an important role in the processes of moulting and metamorphosis, there has been an increasing interest in their study in various groups of insects, and we now possess much information on them in many orders of insects (Williams, 1949). Nevertheless, so far as the Coleoptera are concerned, there are only three records of these structures, and the accounts which they contain differ widely from one another. Arvy and Gabe (1953), while describing the 'cephalic endocrine organs' of *Tenebrio molitor* larvae, mention paired masses of closely aggregated epithelial cells situated in the lateral parts of the head and supplied by nerves from the sub-oesophageal ganglion, and they suggest that these are the equivalents of the prothoracic glands of other Holometabola. They also describe neurosecretory cells in the sub-oesophageal ganglion and believe that their secretory products stimulate the epithelial masses on reaching the latter through the nerves. In the same insect, Stellwaag-Kittler (1954), apparently ignorant of the work of Arvy and Gabe, described the prothoracic glands as slender structures, each running from the vicinity of the prothoracic spiracle along the ventral tracheal trunk supplying the head into the latter, where they attenuate and disappear. Finally, Núñez (1954) gave a detailed account of the prothoracic glands of the carabid, *Anisotarsus cupripennis*. He described them as bands of cells running between and firmly attached to the dorsal and ventral tracheal trunks supplying the head. He also described histological changes which the glands undergo during postembryonic development.

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Such apparent diversity in the accounts of the prothoracic glands of coleopteran larvae indicates the need for a comprehensive study of these structures, and this the present paper seeks partly to fulfil. It contains an account of the glands in the larvae of 15 species of beetles representing 13 families and 8 superfamilies and thus gives a fairly broad picture of the organs in this order.

Material and Technique

The following are the species whose larval prothoracic glands are described here:

Nebria brevicollis (Fab.) (Caraboidea, Carabidae), *Elater rufipennis* Stephens (Elateroidea, Elateridae), *Anthrenus vorax* Waterhouse (Dermestoidea, Dermestidae), *Lasioderma serricorne* (Fab.) (Bostrychoidea, Anobiidae), *Ptinus hirtellus* Sturm. (Bostrychoidea, Ptinidae), *Tenebrio molitor* L. (Cucujoidea, Tenebrionidae), *Oryzaephilus surinamensis* (L.) (Cucujoidea, Silvanidae), *Tenebroides mauritanicus* (L.) (Cleroidea, Trogositidae), *Rhagium bifasciatum* Fab. (Chrysomeloidea, Cerambycidae), *Caryedon fuscus* Goeze (Chrysomeloidea, Bruchidae), *Galeruca tanaceti* (L.) (Chrysomeloidea, Chrysomelidae), *Phaedon cochleariae* (Fab.) (Chrysomeloidea, Chrysomelidae), *Pentarthrum huttoni* Woll. (Curculionoidea, Curculionidae), *Calandra granaria* (L.) (Curculionoidea, Curculionidae), *Araeocerus fasciculatus* (Deg.) (Curculionoidea, Anthribidae).

The study has been based chiefly on dissections under Ringer's solution and on temporary or permanent whole mounts of extracted glands. Because of their extremely small and transparent nature, the glands are not easily visible; the dissection was, therefore, always stained intra-vitally with a dilute solution of methylene blue, which imparts a rather dark colour to the glands.

In all cases the complete or nearly complete glands could be removed only with the dorsal and ventral tracheal trunks of the head intact. To keep the two tracheal trunks attached, the common trunk had to be cut immediately internal to the prothoracic spiracle. All tracheal branches arising from the tracheal trunks were also cut and the two tracheal trunks, together with the gland, were then released from the neighbouring tissue and floated in water. The entire preparation was lifted onto a slide and allowed to remain on it for a few minutes, during which it adhered loosely to the slide. Any rearrangement of structures or necessary manipulations could be done before it is thus set.

Carnoy's or Bouin's fluid was generally used to fix the tissue. Other fixatives, like Flemming's, Flemming's without acetic, Susa, &c., were tried but not found very successful. The fixed material was stained in Delafield's haematoxylin or Heidenhain's iron alum haematoxylin, and sometimes counterstained in eosin. To determine whether the gland was syncytial or cellular, fixed preparations were examined under the phase-contrast microscope. Drawings of the dissections were made with the aid of a squared eyepiece and those of the mounts with a camera lucida. The scales have been indicated separately on each figure.

Observations

Nebria brevicollis (Carabidae).

The prothoracic glands of another carabid, *Anisotarsus cupripennis*, have already been described by Núñez (1954), and those of *N. brevicollis* greatly resemble them. Each gland in *Nebria* is a slender but rather compact, transparent, band-like structure situated in the posterior part of the head and also extending partly into the prothorax (fig. 1, A, *pg*). It has a main or transverse limb stretched across between the dorsal and ventral tracheal trunks, which arise from the prothoracic spiracle and supply the head; this limb is situated immediately behind the level of the dorsal tracheal commissure joining the two dorsal tracheal trunks. It is attached at one end to the ventral tracheal trunk near the point where the latter gives off an inner slender branch to the alimentary canal and nerve-cord and at the other end to the dorsal trunk near the posterior border of the head. When studied carefully, however, the gland is found to run laterally beyond the dorsal tracheal trunk as a very slender band of tissue which joins the muscle-fibres attached to the lateral wall of the prothorax. Somewhere near the middle of the main limb, a conspicuous branch is given off which runs obliquely backwards and laterally. Running across and beneath the dorsal trunk, this also becomes attached to a few slender muscle-fibres arising from the lateral wall of the prothorax. The gland is thicker at the points where it is attached to the tracheal trunks, over the surface of which a few of its cells may also spread. The extent to which these spread depends on the state of development of the gland, but there is never a regular sheath of gland cells around the trachea.

Each gland comprises some 300 to 400 distinct, somewhat polyhedral cells, each containing an ovoid or lobate nucleus measuring approximately 8·6× 7·0 μ (fig. 1, B). No nerve could be traced into the gland, nor does the latter show an intimate connexion with any tracheal branches other than the two trunks already mentioned.

Elater rufipennis (Elateridae).

The prothoracic glands of *E. rufipennis* are situated in almost the same position as those of *Nebria brevicollis*; that is to say, in the posterior part of the head and the anterior part of the prothorax. In this larva, the major part of the brain is located in the prothorax, and so the main prothoracic glands lie one on each side of the brain, nearly at the level of its anterior margin, or sometimes even anterior to it; but the branches and extensions of the gland may also invade the prothorax (fig. 1, C). The main gland is a band- or ribbon-shaped structure containing only one layer of nuclei. Attached at one end to the ventral tracheal trunk, it runs towards the dorsal tracheal trunk, along and closely applied to a thin strand of muscle-fibres (fig. 1, D). On reaching the dorsal trunk, the band runs laterally again along some very slender muscle-fibres and then becomes attached to another branch of the dorsal trunk. From this main body of the gland a few branches may arise, especially near the tracheal trunks; these run in different directions.

The nuclei of the gland are very strongly basiphil and can thus be distinguished easily from the lightly staining nuclei of the tracheal epithelium. They are about 450 in number, ovoid or spindle-shaped, usually arranged

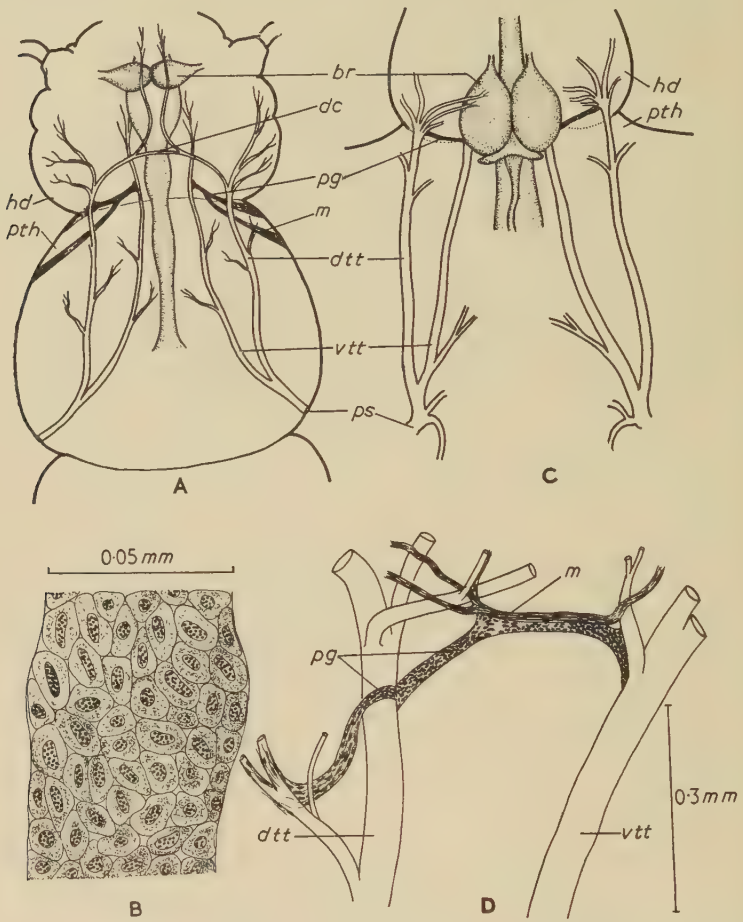

FIG. 1. Prothoracic glands of Coleopteran larvae. A, *Nebria brevicollis*, in natural position (semi-diagrammatic); B, *N. brevicollis*, histological appearance; C, *Elater rufipennis*, in natural position (semi-diagrammatic); D, *E. rufipennis*, gland in relation to dorsal and ventral tracheal trunks.

br, brain; *dc*, dorsal tracheal commissure; *dtt*, dorsal tracheal trunk; *hd*, head; *m*, muscle; *pg*, prothoracic gland; *pth*, prothorax; *ps*, prothoracic spiracle; *vtt*, ventral tracheal trunk.

lengthwise in the gland; they measure approximately $6{\cdot}2 \times 5{\cdot}0\,\mu$. A few nuclei of the gland are also seen on the surface of the muscle-fibres which run in association with it and some may even invade the muscle-bundle. The gland does not, however, spread over the tracheal trunks. Cell boundaries are not visible in the gland, which is an entirely syncytial structure.

nthrenus vorax (Dermestidae).

In the larvae of this beetle, the prothoracic glands are situated in the nterior part of the prothorax or the neck, immediately behind the head. How-ver, as in this case also the major part of the brain lies in the prothorax, the lands are located on each side of it and partly hidden beneath it.

Each gland is a short, wide band of cytoplasm running horizontally across ne two lateral trunks but attached on each side to a major branch of the acheal trunk near its origin and not to the trunk itself (fig. 2, A). It is wider the point of attachment and spreads over the branches arising near the oint of attachment. The gland is a syncytium; there is no trace of cell oundaries. The nuclei are rather large, measuring approximately $14 \times 9{\cdot}2\,\mu$ nd somewhat cuboidal or lobate in appearance; they number about 40 to 50 n each gland.

asioderma serricorne (Anobiidae).

The prothoracic glands of *L. serricorne* greatly resemble those of *Anthrenus* n position and structure. Each is situated in the anterior part of the prothorax little behind the brain, part of which protrudes from the head into the pro-horax. It is a band of syncytial cytoplasm with about 25 large nuclei, attached t one end to a major branch of the dorsal tracheal trunk and at the other to ne ventral trunk immediately before it breaks up into tracheae (fig. 2, B). At ne points of attachment to the tracheae, the gland spreads forwards and back-ards as in *Anthrenus*. The nuclei of the gland measure approximately $2 \times 6{\cdot}1\,\mu$ and, being distinctly larger than the nuclei of the tracheal epithe-ium, can be easily distinguished from the latter.

tinus hirtellus (Ptinidae).

The prothoracic glands of this species also resemble those of *Anthrenus* nd *Lasioderma* in position and structure (fig. 2, C). Each gland is a band of ytoplasm extending from the dorsal to the ventral tracheal trunk and ttached to the trunks near the points where they each break up into tracheae. When well developed, it may be expanded at the points of attachment and ay even appear wound round these, but it does not extend far on the trunks. There are about 20 very large nuclei (measuring about $23 \times 13\,\mu$), which stand a sharp contrast with the much smaller nuclei of the tracheal matrix cells.

enebrio molitor (Tenebrionidae).

The prothoracic glands of *T. molitor* have already been described (Sri-astava, 1958*a*). They are better developed than in any of the other beetles tudied, and are unique in forming a sheath over the dorsal tracheal trunk hen well developed (fig. 2, D). The gland is not syncytial, but is made of umerous cells (800 to 1,000 or more) with comparatively small nuclei like ose in *Nebria*. The nuclei measure approximately $6{\cdot}9 \times 4{\cdot}2\,\mu$ and are only ightly larger than those of the tracheal epithelium; they differ from the latter n staining more deeply with haematoxylin.

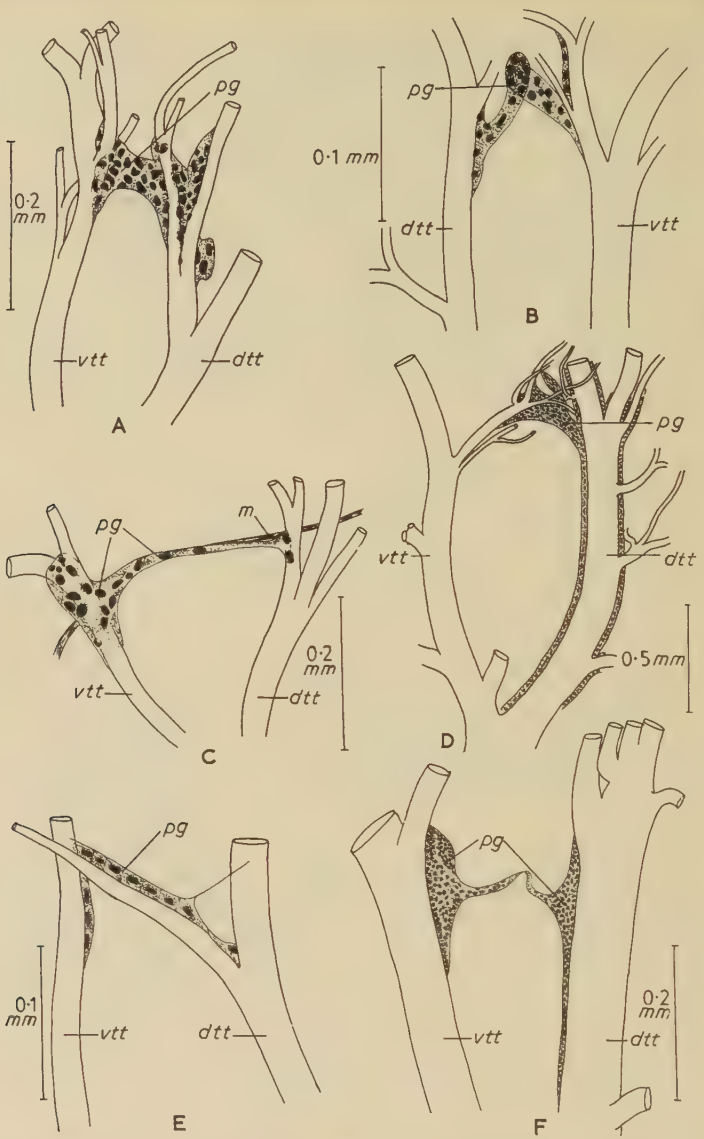

FIG. 2. Prothoracic glands of Coleopteran larvae. A, *Anthrenus vorax*; B, *Lasioderma serricorne*; C, *Ptinus hirtellus*; D, *Tenebrio molitor*; E, *Oryzaephilus surinamensis*; F, *Tenebroides mauritanicus*.
Abbreviations as in fig. 1 (p. 54).

Oryzaephilus surinamensis (Silvanidae).

The prothoracic gland is very slender, like the larva itself. It consists of a row of about 10 to 12 narrow, cylindrical cells attached to the ventral tracheal trunk and running along and closely applied to one of its transverse branches up to the dorsal trunk, to which it is also attached. It is also attached by a thin

ligament-like process, arising from its middle portion, to a branch of the dorsal trunk. In spite of their small size, the cells are much more conspicuous than the very thin tracheal epithelial cells over which they are arranged (fig. 2, E). The gland nuclei are ovoid and measure about $6{\cdot}6 \times 2{\cdot}5\,\mu$.

Tenebroides mauritanicus (Trogositidae).

The prothoracic glands of *Tenebroides* occupy the same position as those of *Tenebrio* but are less extensively developed. The two ends of each gland, which are attached to the tracheal trunks, are considerably expanded lengthwise and thus provide a large area of attachment, while the band running across is very slender. The gland thus presents a typical H-shaped appearance (fig. 2, F). The gland is composed of a number of distinct cells (about 300 to 400), each with a small ovoidal or spheroidal nucleus measuring about $4{\cdot}5 \times 2{\cdot}8\,\mu$. These nuclei are distinctly smaller than those of the tracheal epithelium but are much more strongly basiphil.

Rhagium bifasciatum (Cerambycidae).

In this larva, the prothoracic glands are again situated in the posterior part of the prothorax, a little anterior to the level of the prothoracic spiracles. The two tracheal trunks in this larva soon divide into several thick tracheae and the gland is stretched between a branch of the dorsal tracheal trunk and another of the ventral trunk, as a very delicate strand of cells (fig. 3, A). Compared with the glands of *Nebria*, *Tenebrio*, and also *Anthrenus*, and in proportion to the massive tracheae, the prothoracic gland of *Rhagium* is extremely slender. It is firmly attached to the tracheae and, in a well-developed condition, runs longitudinally backwards as a streak of cells over the surfaces of the two tracheae facing each other. The cells, however, never surround the tracheae completely.

The gland proper is similar in appearance to that of *Tenebroides* and is composed of about 175 to 200 distinct cells of varying shape with spherical or ovoid nuclei. These are larger, measuring approximately $15 \times 11\,\mu$, and stain more deeply in haematoxylin than the nuclei of the tracheal epithelium. Their cytoplasm is intensely granular and also stains well. The gland cells can, therefore, be distinguished easily from the tracheal epithelial cells (fig. 3, B). The cells may often accumulate locally, so that the strand is thicker at some, thinner at other places.

Caryedon fuscus (Bruchidae).

In the larvae of *Caryedon*, each prothoracic spiracle leads, as usual, into the dorsal and ventral tracheal trunks to the head. Another tracheal trunk is also given off anteriorly from the mesothoracic spiracle; this runs forward into the prothorax and unites with the dorsal prothoracic tracheal trunk at about the level of the posterior border of the brain, most of which lies in the prothorax. The prothoracic gland is a well-developed structure wound round the dorsal tracheal trunk at this junction. From the glandular mass thus formed, a band

runs ventro-medially and becomes attached with the ventral trunk where it breaks up into tracheal branches beneath the posterior part of the brain (fig. 3, C). The gland is a syncytium with approximately 500 to 600 ovoidal or spherical nuclei of moderate size, each measuring about $12 \times 8{\cdot}7\,\mu$.

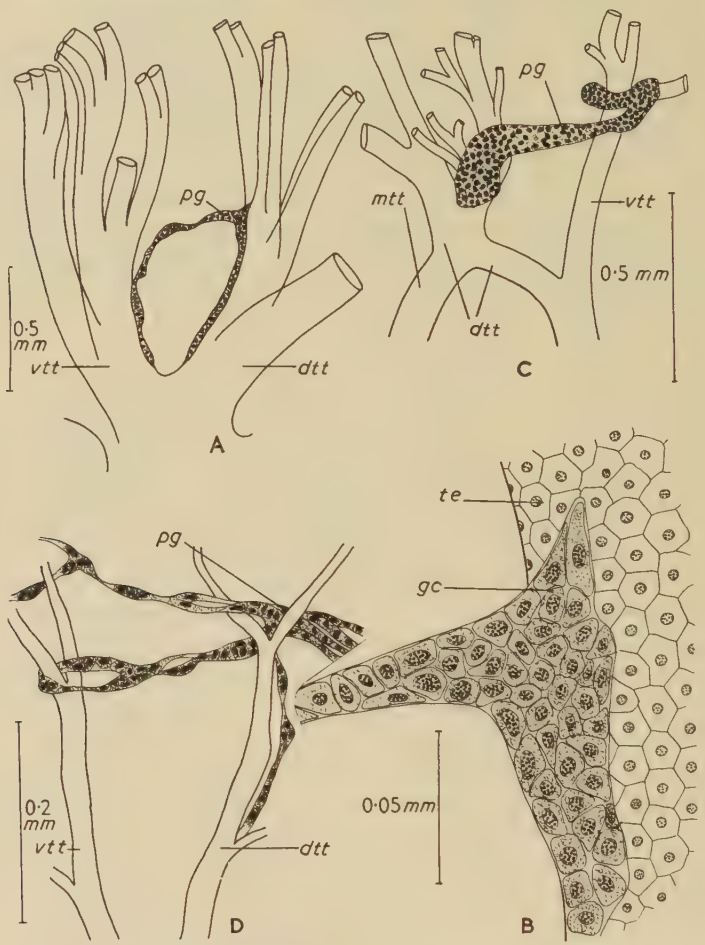

FIG. 3. Prothoracic glands of Coleopteran larvae. A, *Rhagium bifasciatum*, gland in relation to tracheal trunks; B, *Rhagium bifasciatum*, histology of part of gland and tracheal epithelium; C, *Caryedon fuscus*, gland in relation to tracheal trunks; D, *Galeruca tanaceti*, gland in relation to tracheal trunks.

gc, gland cells; *mtt*, mesothoracic tracheal trunk; *te*, tracheal epithelium. Other abbreviations as in fig. 1 (p. 54).

Galeruca tanaceti (Chrysomelidae).

The prothoracic glands of *G. tanaceti* are situated in the prothorax, at about the level of the anterior margin of the prothoracic ganglion. Each gland stretches from the dorsal tracheal trunk to the ventral trunk and is attached approximately at the points of bifurcation of both the trunks into tracheae.

The gland is not compact but very much branched and comprises many strands arising generally from the main transverse band and spread over the tracheal trunks or tracheae; some may pass laterally among fat-body and muscles (fig. 3, D).

The glandular strands consist of 75 to 100 cells; the nuclei measure approximately $6{\cdot}9 \times 4{\cdot}3\,\mu$. Owing to the comparatively large size of the nuclei of the tracheal epithelium, the gland cells do not present a distinct contrast with these, but they can be recognized by their stronger affinity for haematoxylin and by the absence of the tracheal lumen.

Phaedon cochleariae (Chrysomelidae).

In *Phaedon* the prothoracic glands occupy almost the same situation as in *Galeruca*, but are more delicate, very slender, and simpler in nature. As usual, each gland runs between the two tracheal trunks and is attached to them near their bifurcation; it takes the form of a thin strand of about a dozen cells arranged in a row during life and containing large nuclei measuring approximately $12 \times 7{\cdot}4\,\mu$ (fig. 4, A). The strand may, therefore, show a uniform thickness, but owing to irregular shrinkage of cytoplasm on treatment with fixatives, the nuclei may form clumps at some places and may be totally absent at others. The gland may spread over the tracheal trunks a little anteriorly and posteriorly at the points of its attachment with the latter; these extensions may include another 20 to 25 nuclei. Occasionally some delicate strands from this layer may pass laterally among the muscles and fat-body.

Pentarthrum huttoni (Curculionidae).

The prothoracic glands of this species (fig. 4, B, C) are situated far back in the prothorax, which is small but broad and also contains most of the brain. After originating from the prothoracic spiracles, the prothoracic tracheal trunks proceed more or less horizontally for some distance before they take a forward turn. The prothoracic glands are situated a little behind the brain and are, as usual, attached at each end to the two tracheal trunks near the points where each breaks up into tracheal branches.

The gland is in the form of a broad band without branches and ramifications and resembles greatly the prothoracic glands of *Anthrenus vorax* and *Lasioderma serricorne*. It is broader at the two ends for attachment with the tracheal trunks, but much more so towards the ventral trunk. It is syncytial and possesses about 20 to 25 large, darkly staining nuclei, which measure approximately $12 \times 10\,\mu$ and present a great contrast with the small nuclei of the tracheal epithelium. The nuclei may be spherical, elongate, or almost cuboidal, and the cytoplasm is intensely granular.

Calandra granaria (Curculionidae).

The prothoracic glands of this curculionid broadly resemble those of *Pentarthrum* in structure and situation. Each, however, is attached to branches of the two tracheal trunks and possesses 15 to 20 still larger, roughly ovoid

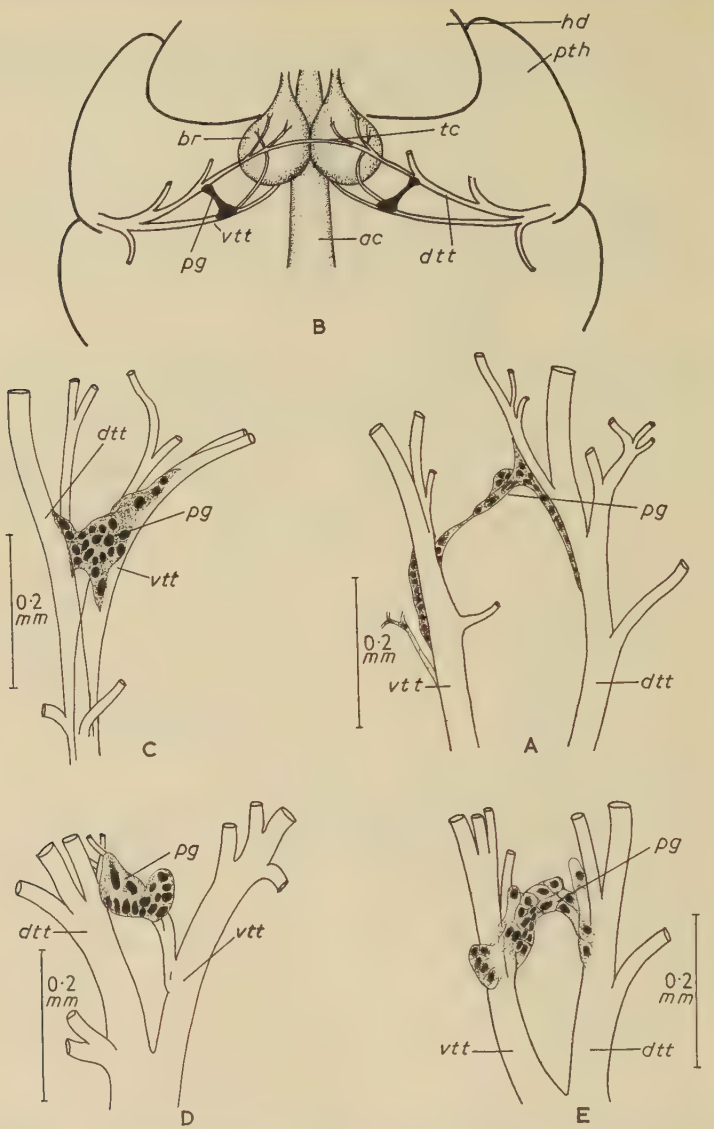

FIG. 4. Prothoracic glands of Coleopteran larvae. A, *Phaedon cochleariae*, gland and tracheal trunks; B, *Pentarthrum huttoni*, in natural position (semi-diagrammatic); C, *P. huttoni*, gland and tracheal trunks; D, *Calandra granaria*, gland and tracheal trunks; E, *Araeocerus fasciculatus*, gland and tracheal trunks.

ac, alimentary canal. Other abbreviations as in fig. 1.

nuclei, which measure about $23 \times 14 \mu$. The gland is a syncytium and is not associated with muscles or nerves (fig. 4, D).

Araeocerus fasciculatus (Anthribidae).

Like the two curculionids, the prothoracic glands of *Araeocerus* are also

situated in the prothorax; they are attached to the two tracheal trunks and partly to the tracheae arising from these. Each is in the form of a broad, somewhat irregular band which differs from the gland of the curculionids in its distinctly cellular nature (fig. 4, E). There are 20 to 25 large cells of very various shapes, each provided with a large nucleus measuring approximately $6 \times 9{\cdot}6\,\mu$.

Discussion

Prothoracic glands and their equivalents, the ventral glands, have been studied in several groups of insects, but surveys sufficiently extensive to justify generalizations on their appearance and structure within any one order or large group have not been attempted except in the Hemiptera-Heteroptera (Wells, 1954). From the facts available, however, it appears that in several orders the glands show an appreciable range of variation. In the Heteroptera, for instance, they may be large, separate, paired, elongated bodies (Wells, 1954) or represented merely by nuclei embedded in the fat-body (Wigglesworth, 1952; Nayar, 1953); in the Lepidoptera they may be either elongate or cobate or branched structures (Fukuda, 1940; Williams, 1947, 1948; Lee, 1948); in the Orthoptera and the cockroaches the glands are usually paired band-like structures running longitudinally across one another from the prothorax into the neck and investing a central core of striated muscles, but in the mantids they are extensively branched and the branches may be distributed along the salivary ducts and elsewhere in the prothorax (Scharrer, 1948; Bodenstein, 1953; Chadwick, 1956). In the Coleoptera, it has been mentioned earlier that there are only three existing accounts of the prothoracic glands. It has been shown elsewhere (Srivastava, 1958 *a*, *b*) that the structures which Stellwaag-Kittler (1954) described as prothoracic glands are the maxillary glands and, contrary to the account given by Arvy and Gabe (1953), the true prothoracic glands are situated in the prothorax and are not supplied by nerves. In fact in the species now examined, and also in *Anisotarsus* (Núñez, 1954), the prothoracic glands show a much greater degree of uniformity in the Coleoptera than in the orders mentioned above, and even species belonging to widely separated families of beetles exhibit comparatively little difference. The present study shows that the prothoracic glands of Coleoptera take the form of a band or string of varying dimensions stretched between the dorsal and ventral tracheal trunks which run from the prothoracic spiracles to the head, or between the branches of these trunks.

Nevertheless, certain minor differences in the position, gross structure, or histology cannot escape attention. In *Nebria brevicollis* and *Elater rufipennis* the glands are situated in the posterior part of the head and extend only partly into the prothorax; in *Lasioderma serricorne*, *Tenebroides mauritanicus*, *Tenebrio molitor*, and the chrysomelids they lie in the anterior part of the prothorax, while in *Rhagium bifasciatum* and the curculionids they are located in the posterior part of the prothorax. It might appear from this that the prothoracic glands are situated anteriorly in the more primitive groups and posteriorly in

the more advanced ones. But Núñez (1954) described the glands of *Anisotarsus cupripennis* within the prothorax, and in the present study the glands of *Rhagium bifasciatum* are found in the posterior part of the prothorax. There can be no doubt that to some extent the larval anatomy determines the precise location of these glands in the prothorax and there is no strict correlation between the systematic position of the beetle and the site of the gland. Still, a clear tendency for the prothoracic gland to be found anteriorly in the primitive groups and posteriorly in the advanced ones can be discerned; and this is quite in agreement with the established fact of the origin of the gland from the second maxillary segment (Toyama, 1902; Wells, 1954).

Notwithstanding other slight differences in their structure, the prothoracic glands of Coleoptera can be divided into two main histological types: cellular and syncytial. Among the 15 species examined, the glands are cellular in *N. brevicollis*, *T. molitor*, *R. bifasciatum*, *O. surinamensis*, *T. mauritanicus*, *G. tanaceti*, and *A. fasciculatus*, and syncytial in *E. rufipennis*, *A. vorax*, *P. hirtellus*, *L. serricorne*, *C. fuscus*, *P. cochleariae*, *P. huttoni*, and *C. granaria*. It is of interest to note that such an important histological distinction is without taxonomic significance, for not only may the same superfamily (e.g. Curculionoidea) but even the same family (e.g. Chrysomelidae) include representatives with syncytial or with cellular thoracic glands. It is not possible to explain the occurrence of such a difference between closely related forms. It may be noted that in the Heteroptera, all members possess syncytial prothoracic glands, while in the Orthoptera the glands have been described as cellular.

From his study of 11 species of Heteroptera, Wells (1954) concluded that the number of nuclei is nearly constant (about 300) throughout the order and that, after making allowance for changes in different phases of development, their size is proportional to the size of the body of the nymph, so that the total nuclear volume of the prothoracic gland bears a constant relation to the total volume of the insect. Such is not at all the case in the Coleoptera. The number of nuclei in this order ranges from about a dozen to about 500 or even more and their size varies from $4{\cdot}5 \times 2{\cdot}8\,\mu$ (*Tenebroides mauritanicus*) to $23 \times 14\,\mu$ (*Calandra granaria*). It may also be noted that the larger beetle larvae do not possess larger nuclei, as found by Wells in the bugs. *Tenebroides* and *Tenebrio*, which are among the largest beetles, possess small nuclei, while *Calandra* and *Ptinus*, although very small, have very large nuclei. Evidently the relationship between nuclear size and insect size noted by Wells does not hold in the Coleoptera. Nor is there any relation between the size of the whole gland and that of the insect. Nevertheless, one cannot fail to notice that in those instances in which the nuclei are numerous they are small, and conversely. *Nebria*, *Elater*, *Tenebrio*, and *Tenebroides* have a large number of comparatively small nuclei, while *Anthrenus*, *Pentarthrum*, *Calandra*, *Araeocerus*, &c., have a smaller number of large nuclei. It is not possible to determine accurately the volume of the whole gland and the total volume of its nuclei, but it appears from these studies that there is a certain rough proportion between the two;

and indeed if the nuclei play an important role in controlling secretion this does not appear surprising.

It seems that the very nature of the prothoracic glands in the Coleoptera—as syncytial sheets of cytoplasm or as bands of cells whose volume is liable to change in different stages of development—and their association with tracheal trunks or tracheae, impart to the glands a tendency to spread over the structures with which they come in contact and result in varying degrees of complexity in their form. In most cases, e.g. the carabids, *Tenebroides*, *Anthrenus*, etc., it is considerably expanded at the points of attachment with the tracheae; in *Rhagium* it forms a ribbon-like extension over one side of the tracheae, while in *Tribolium* it gives rise to a regular sheath over the dorsal tracheal trunk. More interesting, perhaps, is the association of the main prothoracic gland (*Elater*, *Tenebrio*) or its branches (*Nebria*, *Galeruca*) with muscles. In *Leucophaea* Scharrer (1948) describes a bundle of muscle-fibres running throughout the length of the gland and considers that it helps in the discharge of the secretory product, while Wigglesworth (1934) concluded that the stretching of the abdomen of *Rhodnius*, brought about by a full meal, causes the growth and moulting hormone to be discharged. As the muscle-fibres with which the gland is associated in these beetle larvae are attached to the body-wall of the thorax, it is not unlikely that a certain stress on the glands caused by changes in the dimensions of the prothorax may bring about or accelerate the secretory discharge in these cases.

It may be mentioned here that in structure and position, the prothoracic glands of Coleoptera greatly resemble those of the Megaloptera (Rahm, 1952) and that both are quite different from these glands in any other order of insects.

The present observations agree with those of Wigglesworth (1952), Wells (1954), and others in the Hemiptera in demonstrating the absence of nuclear division in the prothoracic glands during postembryonic development. Also, as in the Lepidoptera (Lee, 1948; Scharrer, 1948) but not in the Hemiptera, nerves to the prothoracic glands could not be traced in the Coleoptera.

I am grateful to Prof. O. W. Richards for suggesting this problem and to him, Dr. N. Waloff, and Mr. R. G. Davies for their suggestions and helpful criticisms. I am also grateful to the Committee for Commonwealth University Interchange for the award of a travel grant and to the Government of U.P., India, and the University of Allahabad for financial assistance which enabled me to undertake this work at Imperial College, London.

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The Function of Chromatocytes in the Simuliidae, with Notes on their Behaviour at the Pupal-adult Moult

By H. E. HINTON

(*From the Department of Zoology, University of Bristol*)

With three plates (figs. 2–4)

Summary

In the larvae and pupae of the Simuliidae the cuticle and epidermis of the thorax d abdomen are more or less transparent, and the colour pattern is formed by cells hromatocytes) that contain pigment granules. These cells always lie below the base- nt membrane. It is possible to photograph the chromatocytes without damage to e animal and thus to make photographic records of the behaviour of particular romatocytes over a period of many days when the animal is moving about and feed- g in the normal way.

The chromatocytes accumulate lipids. The accumulation of lipids during larval owth and their depletion during adult development has been photographed in rmal undamaged animals.

Conspicuous changes in the colour pattern, especially of the thorax, occur at meta- orphosis. Such changes are due to mass migrations and the formation of new gregation patterns by the chromatocytes. While such movements of the chromato- es are taking place in some parts of the body, the aggregation patterns of chroma- ytes in other parts of the body remain unchanged and appear to be unaffected by e events that initiate and accompany moults and ecdyses.

Introduction

THE possibility of studying by means of photographs the behaviour of individual cells in undamaged insects that are living normally has always emed remote. Nevertheless, when the cuticle is transparent it is possible to otograph pigmented cells in the body by means of transmitted light with ordinary light microscope. The technique is simple. The larva is removed m the aquarium and treated with carbon dioxide. It is then mounted in a p of water beneath a coverslip in a cavity slide or small chamber. It is erwards returned to the aquarium to recover and grow until the next photo- aph is required.

In larval Simuliidae the general colour pattern of the thorax and abdomen very similar—dark above and paler beneath. The colour pattern is formed cells (chromatocytes) that contain pigment granules. The pigment in the anules is almost certainly no kind of melanin. It can be repeatedly oxidized d reduced by relatively mild agents. The chromatocytes lie below the base- nt membrane (fig. 1). The peripheral layer of chromatocytes usually forms more or less pavement-like dorsal epithelium (figs. 1; 2, A–C; 3, C). This mented layer becomes less close towards the lower sides and is usually

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transformed in gradual stages into an open meshwork on most parts of th ventral surface, where the cells are connected to one another by relativel long, thin or thick, cytoplasmic bridges or pseudopodia (fig. 3, D, E).

The vast majority of the chromatocytes in the body are found in the per pheral layers that are responsible for the general colour pattern. Voinov (1928

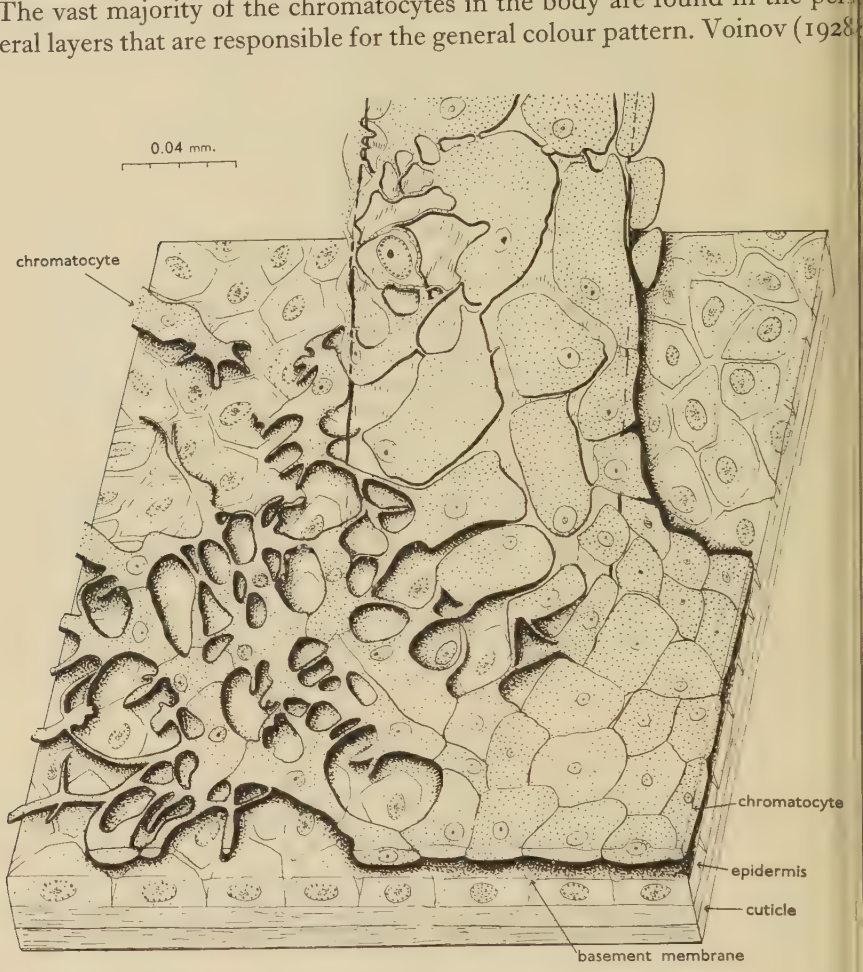

FIG. 1. *S. ornatum*; area of body-wall at the origin of one of the oblique, dorso-ventr abdominal muscles.

believed the chief function of the chromatocytes to be the storage excretio of waste pigments, although she recognized the fact that they also accumulate food reserves subsequently used during adult development. Most aquat larvae have a dark colour pattern above and a paler one beneath. There seem to be no reason to question the general view that the selective value of suc colour patterns is cryptic. In most insects the pigment is deposited in th cuticle, as it is, for instance, in the larvae of the Ephemeroptera, Plecopter and Coleoptera. In many other larvae, however, the cuticle of at least th

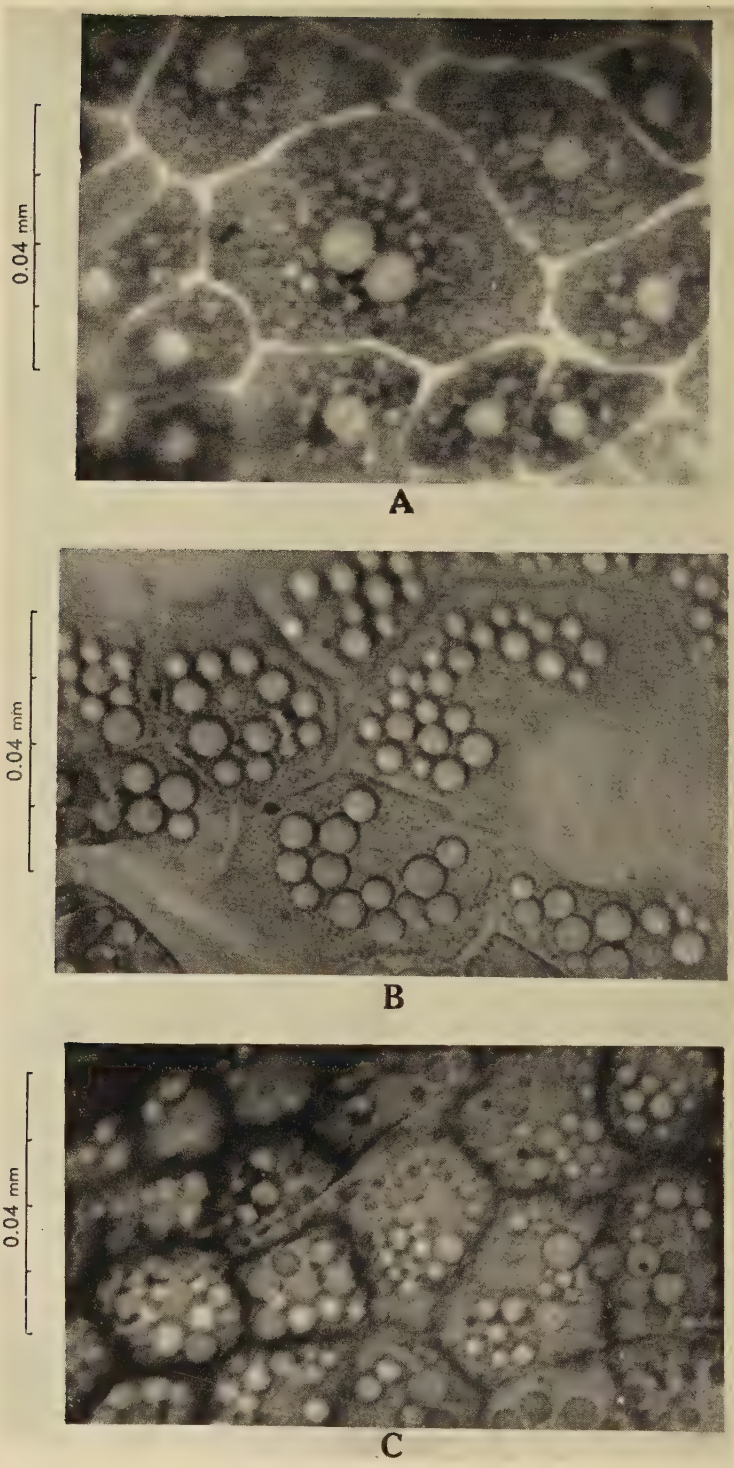

FIG. 2

H. E. HINTON

horax and abdomen is transparent. In such larvae the pigment may be eposited in the epidermis, as in the larvae of many Trichoptera, or it may be eposited in mesodermal chromatocytes, as in the Thaumaleidae (Hinton, 958*d*), perhaps all Simuliidae, and some other dipterous larvae. Simuliid rvae are frequently found on stones and plants in the swiftest parts of reams. They do not leave and hide during ecdyses but shed their cuticles here they are. So exposed, it would seem that a premium is placed upon the ility to shed the cuticle without changing colour. The brain, ventral cord, rger nerves (fig. 3, A, B), and a few of the larger skeletal muscles (fig. 1) are nclosed in a sheath of chromatocytes. The function of these chromatocytes unknown.

Storage of Reserve Food by the Chromatocytes

The chromatocytes of the Simuliidae are probably modified fat-body cells, claimed by Voinov (1928). During postembryonic stages, however, they e easily distinguishable from the usual fat-body cells. In the later larval ages and in the pupal stage they frequently form a partial or complete avement-like or open meshwork, one cell thick, around the larger ribbons sections of the fat-body. The chromatocytes that form such sheaths not ly differ greatly in shape and colour from the normal fat-body cells, but ey are separated from the fat-body by the delicate, non-nucleated sheath connective tissue that envelops at least the larger units of the fat-body.

Besides forming the cryptic colour pattern, the chromatocytes store reserve od. This is stored almost entirely in the form of lipid or lipid-containing obules that colour intensely with Sudan black. The different globules in ch cell are nearly always much alike (fig. 2, B) although they may differ in ze (fig. 2, C). In very young larvae there are usually no or few lipid globules the chromatocytes. As the larvae grow, such globules appear and become ogressively larger. Generally by the fifth instar, or at latest by the early harate pupal stage, most of the chromatocytes are full of lipid spheres. In y stage of development, including the late pharate pupal stage, there is uch variation in the amount of lipid stored in the chromatocytes. In *mulium ornatum*, for instance, there are more or less clearly marked seasonal riations, and, in any particular season, larvae and pharate pupae collected om different streams may differ in their lipid content, presumably because nditions are better for them in some streams than in others.

The chromatocytes (especially those of the peripheral layer beneath the idermis) are rather flattened cells, thickest in the region of the nucleus, the tter causing a distinct bulge (fig. 3, A). The lipid globules therefore tend to arranged in rings around the nucleus (figs. 2, A, B; 3, C, D). In young larvae

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Fig. 2 (plate). Chromatocytes of *S. ornatum*. These and all other photographs are taken rough the integument of the living and uninjured animal.
A, larva 4 mm long, middle dorsal area of eighth abdominal segment.
B, late pharate pupa, ventral side near sixth abdominal ganglion.
C, late pharate pupa, dorsal side of sixth abdominal segment.

that have accumulated little fat (fig. 2, A) or in older larvae that have been starved for prolonged periods (fig. 3, A) the nucleus is particularly easy to see. This is because the pigment granules tend to be fairly evenly distributed through the cytoplasm (fig. 2, B), and the nucleus not only lacks pigment granules but above and below it there is only a thin layer of cytoplasm with comparatively few pigment granules. As the lipid globules increase in size or number or both, the chromatocytes become progressively paler, the nucleus less distinct, and, if the cells are particularly full of lipid spheres, cell boundaries may become difficult to see.

The amount of lipid present in different chromatocytes at any particular time is much the same; no consistent differences have been noted between the pavement-like or branched chromatocytes that form the peripheral layer, nor between these and those that closely invest the brain, ventral cord, and larger nerves.

Changes in the lipid content of easily recognizable groups of chromatocytes in the same individual have been photographed over a period of 8 days, from the early pharate pupal stage to the stage when the adult was nearly fully pigmented (see fig. 4, opposite p. 70). After the pupal-adult moult there is a marked depletion of the lipid reserves of the chromatocytes, a depletion that coincides with the differentiation of the adult and results in an actual decrease in the size of the chromatocytes.

Behaviour of Chromatocytes at the Pupal-adult Moult

The general colour pattern—dark above, paler beneath—is similar in all larval stages of *S. ornatum* Meigen and some other species of the genus. The chromatocytes do not, as claimed by Epure (1937), become larger as the larva grows. To determine the relation between the size of the chromatocytes and the size of the larvae, chromatocytes entirely or partly contained in squares 70 μ by 70 μ were drawn and counted. The results are shown in table 1.

From the figures in table 1 it would appear that the mean size of the chromatocytes is similar in all larval stages. It therefore follows that the number of chromatocytes concerned in the formation of the colour pattern is directly proportional to the surface area of the larva. The range in the size of the chromatocytes is similar in all larval stages. The chromatocytes of *S. costatum* Fried. and *S. equinum* L. are similar in size to those of *S. ornatum*.

As the larva grows the chromatocytes divide and their number increases

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Fig. 3 (plate). Chromatocytes of *S. ornatum*.

A, fourth instar larva collected in the field in January and starved in the laboratory for 7 days. Optical section of chromatocytes around largest nerve from the sixth abdominal ganglion.

B, early pharate pupa 7 mm long collected in the field in May. Optical section of chromatocytes around largest nerve from the sixth abdominal ganglion.

C, larva 5 mm long, middle dorsal area of fifth abdominal segment.

D, early pharate pupa 7 mm long collected in the field in May, ventral side near sixth abdominal ganglion.

E, third instar larva collected in the field in May, ventral side near sixth abdominal ganglion.

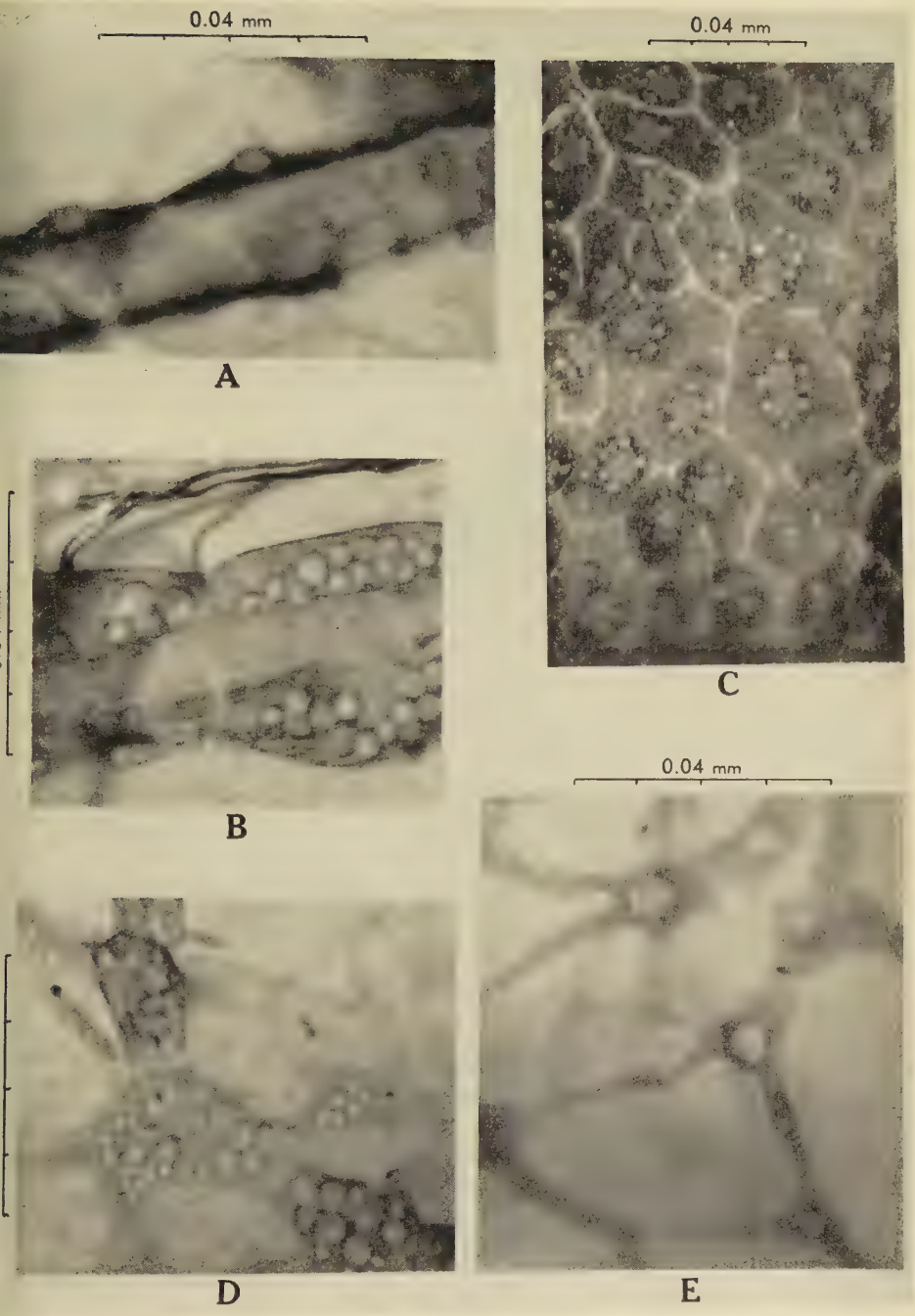

FIG. 3

H. E. HINTON

n postembryonic stages the chromatocytes remain quite distinct from the at-body; increases in their number never appear to be brought about by ecruitment to their ranks from the fat-body cells, as has been claimed by oinov (1928). After maximum surface area of the animal is achieved, all ovements of chromatocytes cease in parts of the body for prolonged periods hich include the larval-pupal ecdysis and the pupal-adult moult. It therefore ppears that in certain parts of the body the chromatocytes are unaffected by e events that initiate and accompany moults and ecdyses. This was estab-shed beyond reasonable doubt by making photographic records of groups

TABLE 1

Length of larvae and number of chromatocytes

No. of larvae used	*Length of larvae in mm*	*Number of chromatocytes in squares 70μ × 70μ*		
		Minimum	*Maximum*	*Mean*
7	1·3	8	15	12·0
20	7 to 9	5	20	12·3
62	1·3 to 9	4	25	12·4
24	4 to 9 (central nervous system only)	6	25	12·4

f chromatocytes in the abdomen from long before to long after the pupal-dult moult.

The pupal-adult moult was selected for study because no great changes in rface area occur at this time. The posterior ventral part of the abdomen was hosen because in this region the skeletal muscles of the pupa are carried over ith relatively little change into the adult stage. The necessity for choosing a art of the body where changes in the skeletal musculature are slight arises ecause chromatocytes cannot be present between a muscle and the area of e cuticle in which it is inserted. Therefore, as has recently been pointed out Hinton, 1958*a*), great changes in the disposition of the skeletal muscles ecessarily impose great changes in the pattern of chromatocytes. Thus the normous differences between the thoracic musculature of the pupa and dult impose a more or less complete change in the thoracic pattern of hromatocytes between the time that the larval cuticle is shed and the time hat the muscles are inserted in the new adult cuticle. The changes in the keletal musculature of the thorax between the pupal and adult stages could ot be successfully completed without mass migrations of the chromatocytes nd the formation by them of new aggregation patterns, but nothing is yet nown of the factors that initiate these migrations and bring them to an end.

The most successful series of observations and those which involved the ngest time interval were made on a specimen of *S. equinum* L. Photographs f the ventral chromatocytes on each side of the last three abdominal ganglia f this specimen were taken over a period of 8 days, from the early pharate

pupal stage to the time when the adult was nearly fully pigmented (fig. 4, A–F). For the sake of brevity, only the observations on this specimen will be described in detail. The photographs were taken at the following times: A, at 1 p.m. on 8 February; B, at 11 a.m. on 9 February; C, at 9.30 p.m. on 10 February; D, 2 min. after E; E, at 6.45 p.m. on 11 February; F, at 12.45 p.m. on 16 February (see legend to fig. 4).

The first photograph (fig. 4, A) was taken at least a day after the larval-pupal moult. The Simuliidae are unusual amongst endopterygote insects in that the larval-pupal moult is separated from the larval-pupal ecdysis by an interval of several days (about 5 days at 10° to 13° C), during which time the pupa feeds actively and behaves like the preceding larval stage: the pupa stops feeding and spins its cocoon only an hour or so before it sheds the larval cuticle (Hinton, 1958*b*, *c*). After the second photograph (fig. 4, B), the larval cuticle was accidentally pricked opposite the junction of the seventh and terminal abdominal ganglion. The black wound-plug subsequently produced is evident in the third photograph (fig. 4, C). At the time of the latter photograph, the recurved abdominal hooks of the pupal cuticle had become sufficiently sclerotized to be plainly visible beneath the larval cuticle. These are the hooks that engage the fabric of the cocoon when the larval cuticle is shed. When the animal is bred at 10° to 13° C, the hooks are first visible 19 to 20 h before the larval cuticle is shed (Hinton, 1958*b*). However, the specimen now concerned was kept at a slightly higher temperature, sometimes rising to as much as 16° C. For this reason fig. 1, C was probably taken not more than about 12 h before the larval cuticle was shed. The fourth photograph (fig. 4, D) was taken about 2 min *after* fig. 4, E. The fifth photograph (fig. 4, E) was taken 21 h after the third and after the larval cuticle was shed, perhaps about 12 h after this ecdysis. The recurved hooks on the pupal abdomen are now plainly visible: the ventral system of hooks consists on each side of two hooks close together on the fourth abdominal segment and two very widely separated ones on the fifth and sixth segments. The outer member of the two posterior pairs of hooks lies outside the field of the last two photographs. The last photograph (fig. 4, F) was taken when about two-thirds of the pharate adult stage had been completed and the adult was fully pigmented. The specimen was accidentally injured shortly before emergence, and no further photographs were taken of it.

When due allowance is made for slight differences in focal level and for the

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FIG. 4 (plate). Ventral view of posterior abdomen of *S. equinum* photographed at different times during an 8-day period.

A, pharate pupa not less than 24 h after larval-pupal moult.

B, pharate pupa 22 h after A.

C, pharate pupa 56 h 30 min after A.

D, pupa or very early pharate adult about 2 min after E.

E, pupa or very early pharate 77 h 45 min after A.

F, pharate adult 191 h 15 min after A. The pharate adult is now nearly fully pigmented.

a, *b*, *c*, easily recognizable groups of cells to be seen in A to F; *h*, recurved hooks on fourth abdominal segment of pupa; *k*, recurved hooks on fifth abdominal segment of pupa; *m*, recurved hooks on sixth abdominal segment of pupa; *t*, trachea between epidermis and layer of chromatocytes; *w*, wound-plug on larval cuticle.

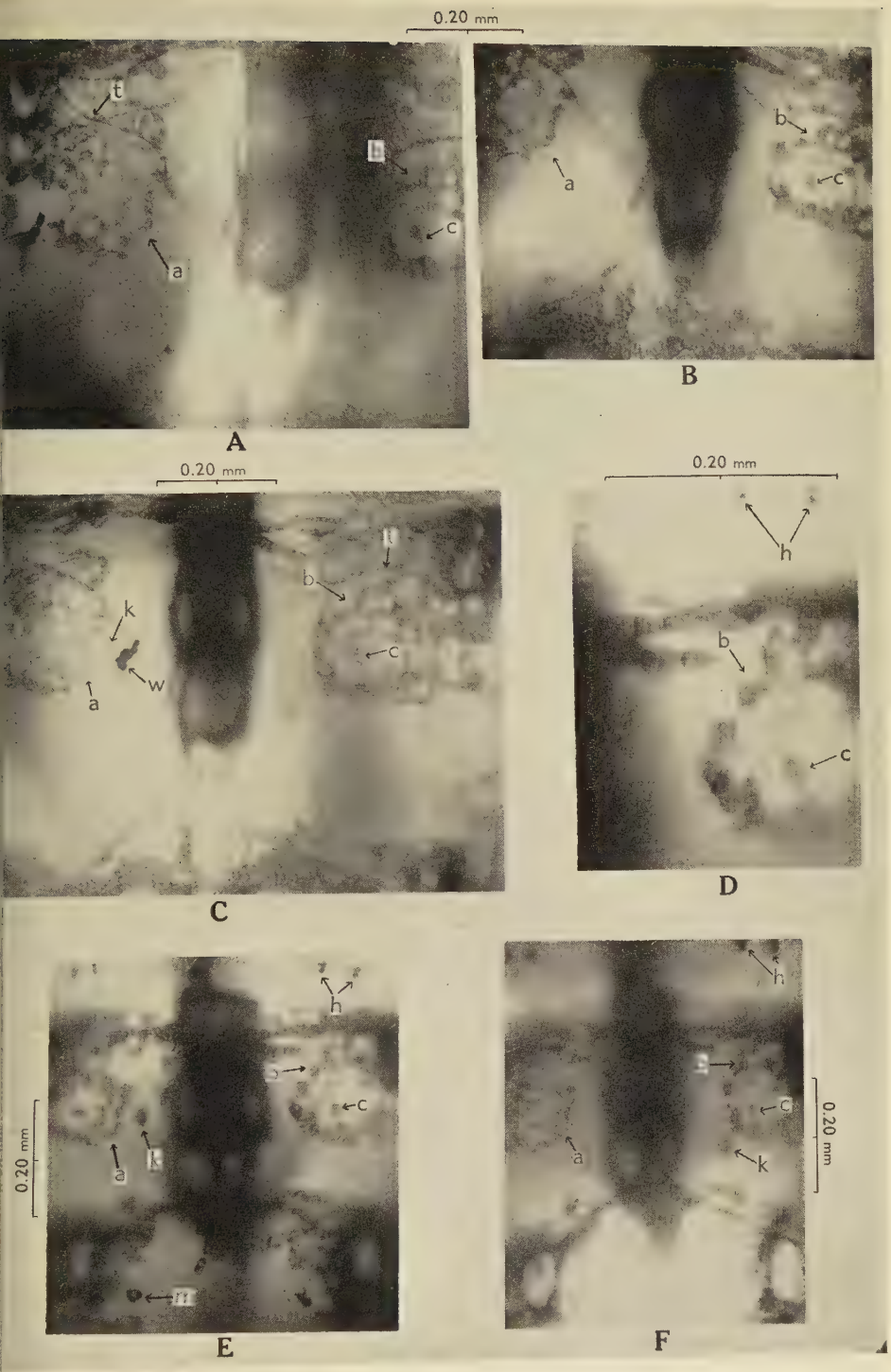

FIG. 4

H. E. HINTON

pletion of lipid reserves and a decrease in size of the chromatocytes, it ould appear (compare especially cell group *a* in A to E, fig. 4) that certain oups of chromatocytes underwent no real changes in position relative to one other for 8 days, a period which included both the larval-pupal ecdysis and e pupal-adult moult.

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The Histology of the Tube-feet and Clavulae of *Echinocardium cordatum*

By DAVID NICHOLS

(*From the Department of Zoology and Comparative Anatomy, University Museum, Oxford*)

With two plates (figs. 2 and 5)

Summary

The histology of the oral feeding, dorsal funnel-building, sub-anal, sensory, and respiratory tube-feet and the clavulae of the fascioles of *Echinocardium cordatum* is described. The penicillate tube-feet, all superficially similar, are shown to possess histological differences which reflect their function: the burrow-building tube-feet (dorsal and sub-anal), which plaster mucus on to the burrow walls, possess mucus glands surrounded by a basket work of muscle-fibres for more efficient discharge, whereas the feeding tube-feet, needing a sticky disc, have glands lacking such fibres. The sensory tube-feet lack definite discs but possess greatly enlarged subepidermal nerve plexuses. The respiratory tube-feet have a thin, much-folded wall; no nervous tissue was detected and no muscle-fibres. Each clavula has two longitudinal bands of cilia, opposite each other, on the stem and a distal knob in which mucus glands are embedded.

Introduction

UNLIKE those of the regular echinoids, the tube-feet of the irregulars, particularly members of the order Spatangoida, show considerable division of labour related to their burrowing habit. The positions of the various tube-feet of *Echinocardium cordatum* (Pennant) are shown in fig. 1. The way these tube-feet are used during burrowing has been described elsewhere (Nichols, in press). In brief, this spatangoid burrows to a depth of up to 8 cm in sand, building a respiratory funnel from its burrow to the surface of the substratum by means of 70 or so very extensile tube-feet borne by the adapical plates of the anterior ambulacrum (fig. 1). A backward prolongation of the burrow, for removal of waste including the respiratory water, is built by similar extensile tube-feet arising from the sub-anal region. The animal feeds mainly by picking up particles of the sand on the sticky ends of a third type of tube-foot, about 40 of which arise in the phyllodes round the mouth. The funnel-building, sub-anal, and feeding tube-feet are all penicillate: their discs are either covered or fringed by finger-like papillae. Although a certain amount of gaseous exchange undoubtedly occurs across the walls of all the tube-feet, there is a region in the dorsal part of each of the lateral ambulacra where the tube-feet are modified solely for respiration, and here they are flattened and folded. Lastly, the regions of each ambulacrum between those mentioned above, i.e. in the ambital parts of each ambulacrum and in the periplastronal areas of the posterior pair as well, bear much reduced tube-feet

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whose function appears to be wholly sensory, since they can be seen extending slightly during burrowing to explore the surrounding substratum.

Currents on the surface of the urchin for respiration, sanitation, and some of the feeding are produced by cilia, which occur over the whole anima

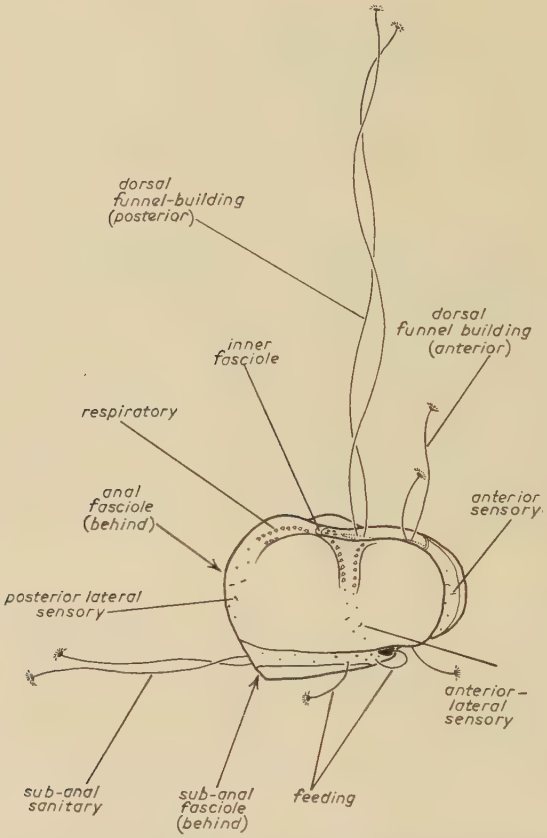

FIG. 1. Diagram showing the positions of the various tube-feet and clavulae (the spines of the fascioles) on the test of *E. cordatum*. A few of each group of tube-feet are drawn extended the others are represented by their pores.

except the older spines. These currents are augmented by those produced a the fascioles, which are special bands of highly ciliated spines called clavulae The fascioles occur in three places in *E. cordatum* (fig. 1): on the dorsa surface (inner fasciole), just below the anus (sub-anal), and surrounding th anus (anal).

The tube-feet and clavulae are thus vitally important in burrowing. Durin this study considerable corroboration has been gained of their function, eve to the extent of supplying information as to their mode of operation no readily obtainable by direct observation.

The histology of these structures has been only cursorily described pre

busly (e.g. Perrier, 1870; Hamann, 1887; Ludwig and Hamann, 1902). me of the tissues have been misidentified in the past, and others ignored; e unique nature of the mucus-secreting epithelium of the burrow-building be-feet has not been previously reported, nor has the histology of the ampulla ll.

Material and Methods

In this work active animals were killed for material, because the tube-feet echinoids often become necrotic even before the animal ceases all move- ent. In most cases the tube-feet were cut off close to the test, but in some, rticularly where the ampulla was examined or the tube-foot was minute, small portion of the test was cut away, enclosing the tube-foot and its npulla. This was done for examination of the clavulae also, in order to keep tuft together during preparation.

The fixative used almost entirely was Heidenhain's 'Susa', made up in sea- ater, with subsequent dehydration in 96% alcohol made light brown with dine (Pantin, 1948). The trichloracetic acid in the fixative decalcified the ssue efficiently, the minimum fixation time being about 6 to 8 h. The best ctions were obtained after embedding by Peterfi's collodion-paraffin method eterfi, 1921), in which the decalcified tissues are impregnated with 1% llodion in methyl benzoate to preserve their shape, and subsequently em- dded in wax (m.p. 58° C). This method gave excellent ribboning for serial ctions, the sections being cut at 6 μ.

The main staining technique employed was Masson's trichrome, with ansen's trioxyhaematein as a nuclear stain. This was particularly suitable, cause in most cases the mucus within the mucus glands took up the light een, and it differentiated particularly well between muscular, nervous, and nnective tissues. Other stains used were:

1. Heidenhain's iron haematoxylin, for general cytological detail;
2. toluidine blue, thionine, and Southgate's mucicarmine for mucus;
3. orcein for elastic tissues;
4. Curtis's substitute for van Gieson, for collagen (Carleton and Drury, 1957).

The Dorsal Funnel-building Tube-feet

The ampulla. The dorsal funnel-building tube-feet of *E. cordatum* are its ost extensible tube-feet. Their ampullae are correspondingly larger than ose of any other, and provide suitable material for the examination of this rgan, the histology of the muscles of which throws considerable light on e muscles elsewhere in the tube-foot system. Brief examination of other mpullae has shown that they do not differ appreciably, and therefore these ill be described as representative.

From sections (fig. 2, A) it can be seen that the ampulla is bounded by a in epithelium, made up of cells containing small round nuclei about 3μ in iameter. On the free border of these cells is a thin cuticle, not more than $\frac{1}{2}$ μ ick. Inside this are two layers of connective tissue, an outer diffuse layer,

some 3–4 μ thick, and an inner denser layer, about 1·5 μ thick. The rest of the wall of the ampulla, except for a very thin coelomic epithelium, consists of muscle-cells making up by far the greater part of the wall. The muscle fibres lie in a layer internal to the dense connective tissue layer, with the greater part of their cell-bodies internal to this again. From sections alone it is not easy to determine the relation of the cell-bodies to the muscle-fibres but a whole mount, suitably treated, shows this clearly.

FIG. 3. Schematic diagram showing the structure of the muscle-tissue of a tube-foot ampulla drawn as though the fibres had been pulled apart laterally and the epithelia removed. Each fibre consists of two bundles of between 4 and 7 fibrils (7 have been drawn in each case) twisted round each other. Those fibres to the left of and including fibre A have been drawn with their cell-bodies in place; the cell-wall has been partly removed in fibre B and completely removed in fibre C to show the spiral structure; in fibre D the cell-wall is in place but the cell-bodies have been removed.

A whole ampulla was removed from the animal, teased, and then stained with Masson's trichrome. This showed that each muscle-cell consists essentially of a cylinder of cytoplasm enclosing two bundles of muscle-fibres which twist round each other. At irregular intervals the fibres anastomose, connexions passing between adjacent bundles, so that a complete network is formed (fig. 3). A lobe of cytoplasm containing the nucleus bulges from that part of the cell containing the muscle-fibres into the water-vascular cavity, the

FIG. 2 (plate). A, section of part of an ampulla wall from the dorsal part of the anterior ambulacrum. All sections stained with Masson's trichrome.

B, longitudinal section through a dorsal funnel-building tube-foot. The section is not quite median, but passes through the origins of two papillae.

C, enlarged view of part of the disc of B. Surface of the disc towards the bottom.

D, section through part of the disc and one papilla of a dorsal funnel-building tube-foot showing the annular plexus giving off nerve branches to the papilla and the disc.

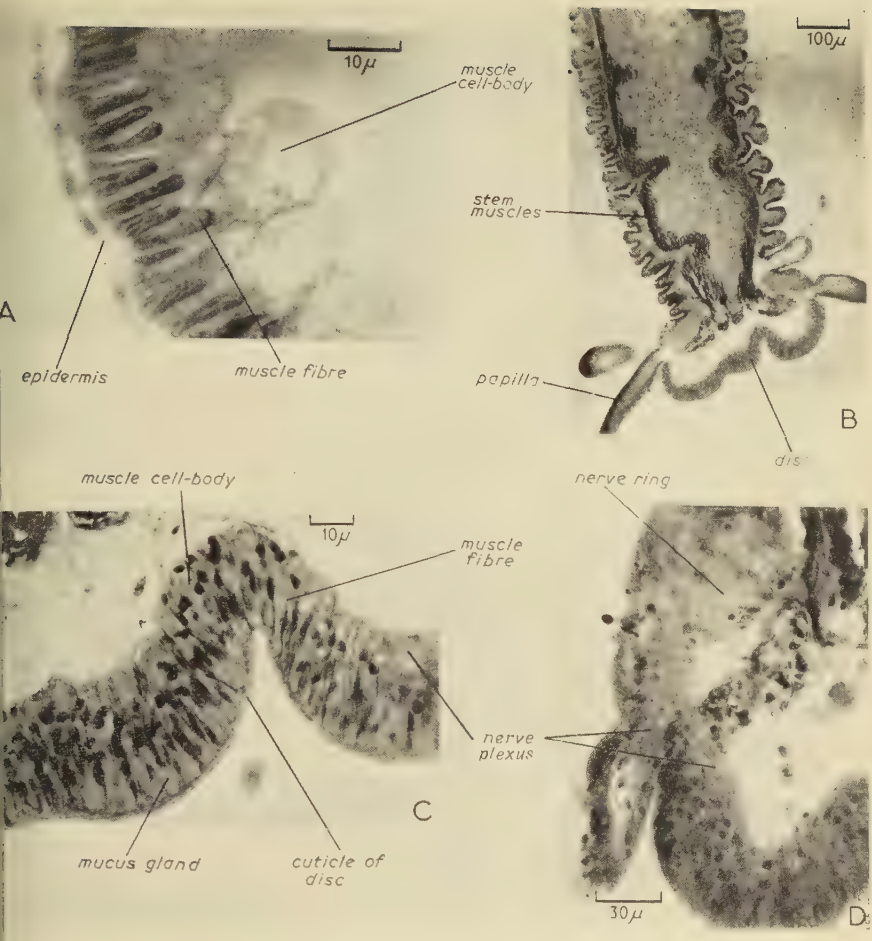

FIG. 2

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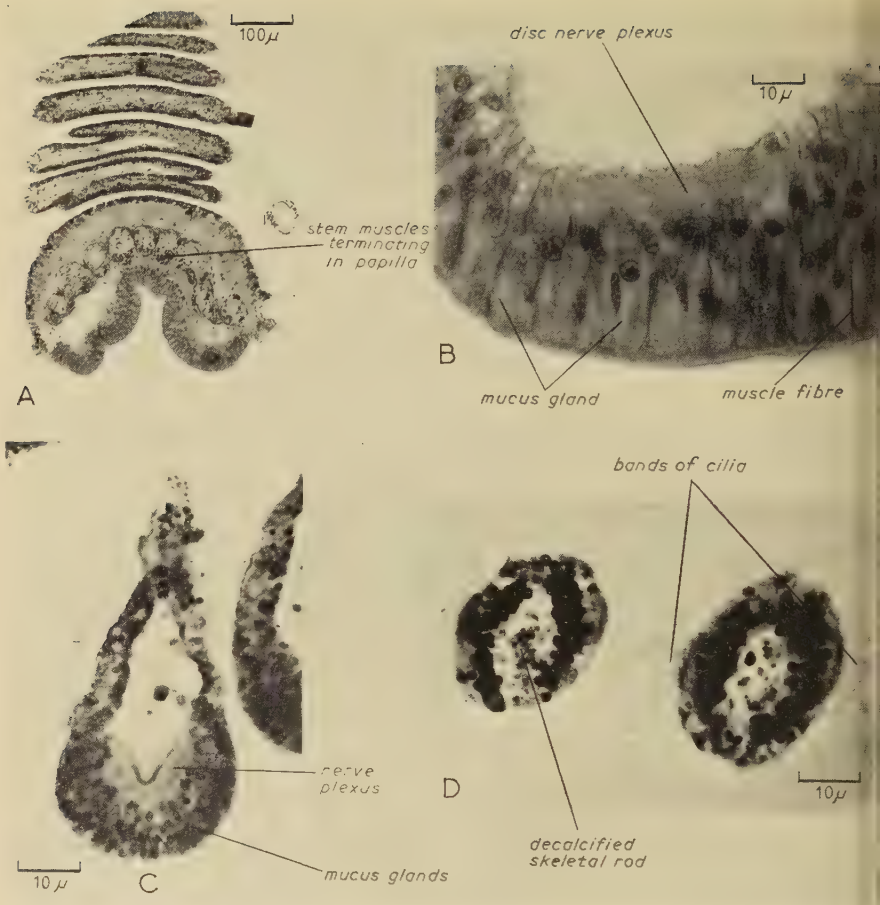

FIG. 5

D. NICHOLS

elomic epithelium forming a thin layer internal to the layer of lobes. The asson technique is particularly suitable for this tissue, because the cell-alls take up the light green and can be seen between the bundles of muscle-pres; however, it is impossible to see cross-walls between two adjacent cells the same bundle, and it is likely, therefore, that the tissue is a syncytium. urther evidence of this may be inferred from macerated tissue. A number fresh ampullae were placed in Goodrich's dissociator (Goodrich, 1942), ft for various periods up to 45 days, and subsequently stained in Mayer's emalum. Partial dissociation occurred after 2 days; that is to say, the epi-helial cells and connective tissue separated from the muscle-tissue, but the uscle-cells remained together in a sheet. Even after 45 days no further paration had occurred.

The nature of the granular material in the lobe of the muscle-cell was amined in sectioned material. This gave a positive PAS reaction, with suit-ole controls, in some of the more deeply situated lobes, but after incubation saliva for 45 min at room temperature similar cells gave a negative PAS action. These observations strongly suggest the presence in the muscle-cell toplasm of glycogen as a food store. Pearse (1954, p. 438) suggests that eriodic acid removes a large amount of glycogen from unprotected sections, hich might explain why most of the more superficial cells of the tissue, after araffin embedding, were negative, even though deeper ones in the same ssue were positive.

The coelomic epithelium in the ampulla is exceedingly thin, and in some aces can only be detected by the presence of its nuclei, which are mostly al, about 5 μ long and 3 μ wide.

To summarize, the muscular tissue of the ampulla is arranged with the undles of fibres in a layer external to the main bodies of the cells containing ie nuclei; these glycogen reservoirs bulge into the cavity of the water-ascular system. I am aware of no previous description of the histology of a ube-foot ampulla, except that of Smith (1947), who dealt only with the nervation of this organ.

The stem. In section (figs. 2, B; 4) the stem shows the following clearly elimited layers:

1. An outer epidermal layer bounded by a thin cuticle. This layer, heavily igmented and with many nuclei (4 to 5 μ in diameter), is about 20 μ thick nd transversely folded when the tube-foot is relaxed. Most of the nuclei nd the pigment granules are crowded towards the external surface (fig. 2, B),

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FIG. 5 (plate). A, L.S. through the edge of a disc of a dorsal funnel-building tube-foot, owing the origins of some 11 papillae and the continuations of the stem musculature within em.

B, L.S. through part of the disc of a dorsal tube-foot, showing the nerve plexus which mifies between and above the inner cells of the epithelium.

C, L.S. through the terminal knob of an oral feeding tube-foot papilla, showing the con-ntration of mucus-glands, of which only the nuclei are visible, and the subepidermal nerve lexus.

D, T.S. of the stems of two adjacent clavulae.

the rest of the layer being somewhat diffuse and consisting mainly of transversely running connective tissue fibres (the *Stützfasern* of German authors).

2. A thin (1–2 μ) layer of nerve-tissue lying immediately beneath the epidermis. This is continuous round the whole of the stem, but if the plane of longitudinal section has been taken through the longitudinal nerve, thi

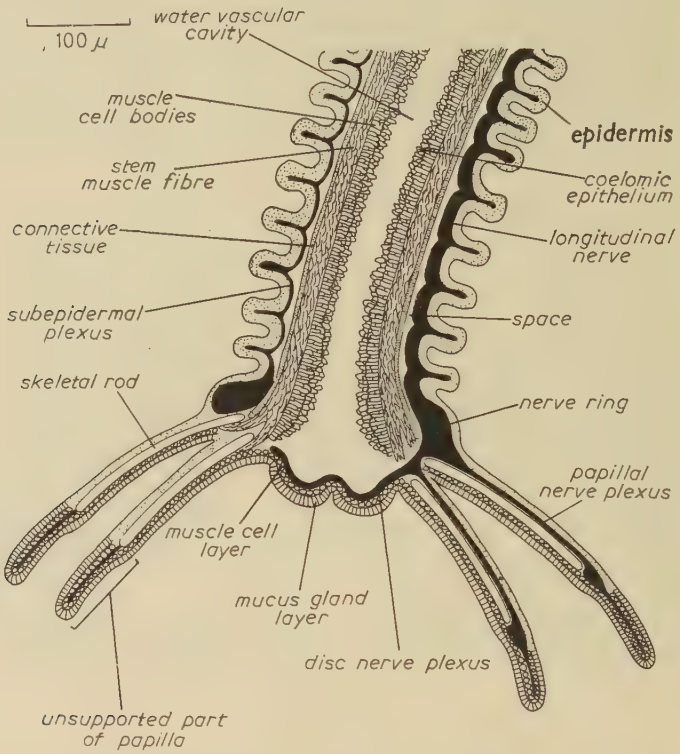


FIG. 4. Diagram of a L.S. through a dorsal funnel-building tube-foot. The section has been taken through the longitudinal nerve-tract on the right side of the stem, and also on this side the nerve-supply to the papillae and disk is shown. On the left side the attachment of the stem musculature to the skeletal rods of the papillae is shown.

appears as a much thicker plexus on one side of the stem, as in fig. 4, right-hand side. The longitudinal nerve is normally 20–30 μ thick. Between the nerve-layer and the next layer there is a space, possibly continuous with the perihaemal canal system, to allow the contractile elements in the centre of the stem to operate independently of the epidermis.

3. A double layer of connective tissue. This has a similar pattern, and presumably function also, to that described by Smith (1947, pp. 287–9) for the asteroids.

4. Internal to this is the band of muscle-fibres some 20 μ thick. In the relaxed state in which the tissues are sectioned these appear as loops. The cell-bodies of these muscles appear to have a similar arrangement to those o

e ampulla, forming a layer internal to the layer of fibres. These are more fficult to interpret as muscle-cell-bodies than those of the ampulla, since th they and the muscle-fibres are more crowded, but in transverse section e cell-walls of some of them can be followed between adjacent fibres. These ll-bodies also gave reactions with the PAS reagent, indicating that they con- n glycogen.

5. The innermost layer, the coelomic epithelium, is much more noticeable an in the ampulla, although in some sections it is difficult to distinguish e line where the muscle-cell-bodies end and the coelomic epithelium begins. general, however, the cells of the epithelium are much smaller, and so are eir nuclei (4 μ in diameter). In places the layer is more than one cell thick.

The histology of the stem differs very little in the other tube-feet of *E. rdatum*, except, of course, those for respiration, which are non-extensile.

The disc and papillae. The general arrangement of the tissue in the disc d papillae can be seen in figs. 2, B, C, and 4. At the distal end of the stem e subepidermal nerve-plexus thickens to form an annular plexus, which es off branches to each papilla and sends ramifications over the inside the disc (fig. 2, D; 4); some fibres of this plexus lie internal to the cells of e epithelium while others ramify between the cells. The muscle-fibres of e stem terminate at the slightly arcuate calcite rods supporting the papillae e left-hand side of fig. 4 and fig. 5, A), while the connective tissue layer of the m terminates just short of the proximal ends of these rods. The unspecialized idermis of the stem continues on to the outside edges of the papillae as far the distal ends of the calcite rods, where it gives way to a highly specialized ucus-secreting tissue which covers the unsupported ends of the papillae, e whole of their inner surfaces and the whole of the disc.

This mucus-secreting epithelium (fig. 6), very closely associated with the rve plexus of the disc and papillae, consists in the main of two distinct ers:

1. The outermost layer consists almost entirely of large, conspicuous, sk-shaped mucus-glands up to 25 μ long and 6 μ wide, which form a arly continuous sheet. The contents of the glands appear very faintly anular in fixed preparations, and stain very slightly with the light green of asson's trichrome. They can give a fairly intense γ-metachromasia with luidine blue or thionine. Their long thin nuclei usually lie about half-way ng the sides of the cells, and are 4–6 μ long, 2 μ wide. The surface of the sc, external to the mucus-glands, is bounded by a cuticle about 1 μ thick, erced by the ducts.

2. The layer internal to the mucus-glands consists of the cell-bodies of uscle-cells which lie either singly or in groups separated from each other elements of the nerve plexus. Fig. 2, C shows in the centre a section of the sc-wall cut through the muscle-cell bodies while on the right-hand side a rt of the nerve plexus is in the plane of the section. In fig. 5, B the section s been taken through a dense tract of the disc plexus, though parts of the uscle-cells, including the round nuclei, can be seen external to (below) the

plexus. From serial sections it can be seen that there are as many connective from the annular plexus to the disc plexus as there are papillae, the connec tives running between the origins of the papillae; on the disc itself the branches ramify extensively.

The contractile elements of the muscle-cells extend from their cell-bodi

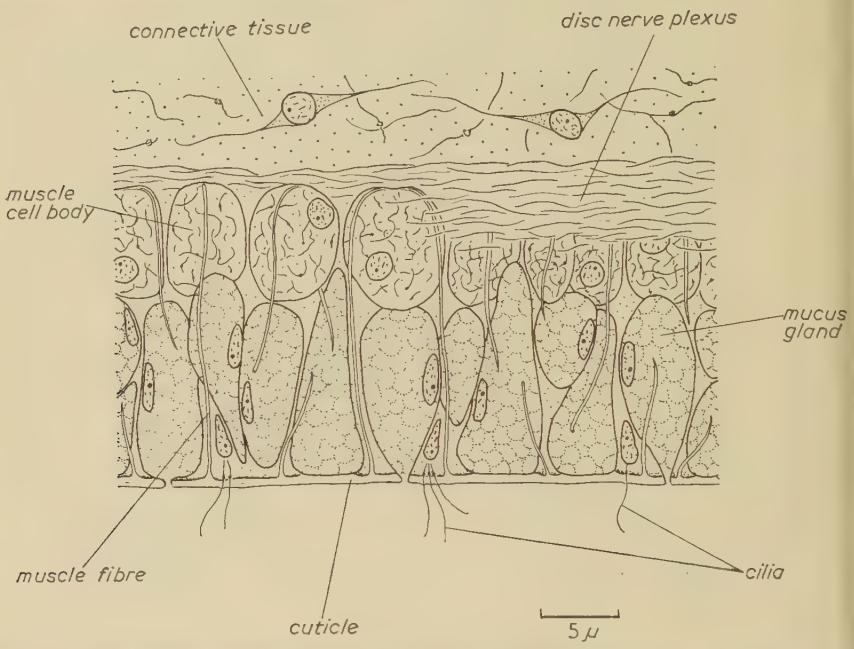


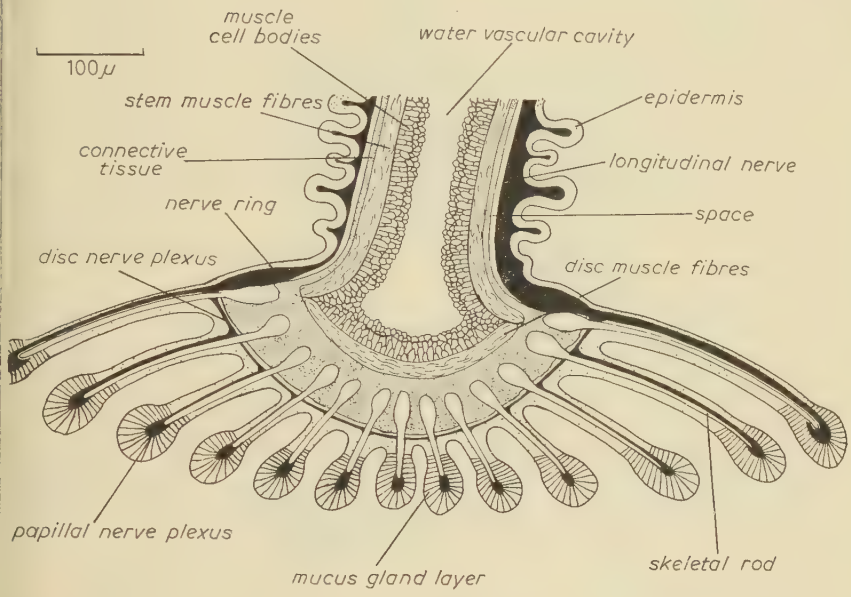
Fig. 6. Vertical section through the specialized epithelium of the funnel-building tube-fee The nerve plexus ramifies between blocks of muscle-cells and part of it forms a sheet interna to the epithelium.

to the cuticle bounding the disc and papillae, passing between the mucus cells (figs. 2, c; 6). There are approximately 4 fibres round each mucus-glan and probably the same number arise from each muscle-cell-body, though th thinness of the cell-walls makes this difficult to ascertain directly. An approxi mate count showed that there are about as many muscle-cells as mucus glands, and it is possible, therefore, that each gland has its own unit o muscles surrounding it, though this is not necessarily a prerequisite for th efficient operation of the glands.

The only other cells which can be detected in this specialized epitheliun are ciliated cells. The nuclei of these cells, either long and thin like those o the mucus glands or triangular, lie very close to the cuticle, and give off ver small cilia (about $\frac{1}{4}$ μ thick) which can be seen to pass through the cuticl to the outside. Numbers of these can be seen in fig. 5, b. Their basal granule are occasionally visible, lying just inside the cuticle.

The Oral Feeding Tube-feet

Unlike the dorsal funnel-building tube-feet, these for feeding have a disc uch wider than the stem (compare figs. 6 and 7). Where it widens out the bepidermal nerve plexus enlarges to form a nerve-ring. The disc plexus anches from this and supplies further branches to each papilla, terminating a thick plexus beyond the distal end of each skeletal rod (fig. 5, c). The oximal ends of these rods lie embedded in connective tissue which, though



G. 7. Diagram of a L.S. through an oral feeding tube-foot. The section has been taken rough the longitudinal nerve tract of the stem on the right, and through 13 of the 250 or so papillae which arise from the disc.

is a continuation of the stem connective tissue, is much less dense. A feature the disc histology not present in the funnel-building tube-feet is a layer of uscle-fibres with their associated cell-bodies lining the inside of the disc g. 7).

The knobs of the papillae (fig. 5, c) are heavily pigmented, and consist most entirely of single-celled mucus-glands, 15–18 μ long and 3–4 μ wide. hese do not take up the light green of Masson's trichrome, but stain bril- ntly with mucicarmine, and exhibit faint γ-metachromasia with toluidine ue or thionine. No muscle-fibres surround the mucus-glands. Cilia can be en passing through the cuticle to the exterior; these are probably less than μ thick, at just about the limit of light resolution. Here again the nuclei of e ciliated epithelial cells lie nearer the cuticle than those of the mucus- ands.

The Sub-anal Tube-feet

The histology of the stem (fig. 8) is very similar to that described for th dorsal funnel-building tube-feet (p. 77 above). That of the disc is superf ficially similar to the feeding tube-feet in that the disc has a covering rathe than a fringe of papillae. However, the papillae at the centre are shorter tha those round the edge (fig. 8), and their calcite supporting rods are not em bedded in the diffuse connective tissue of the disc, but arise on the dista

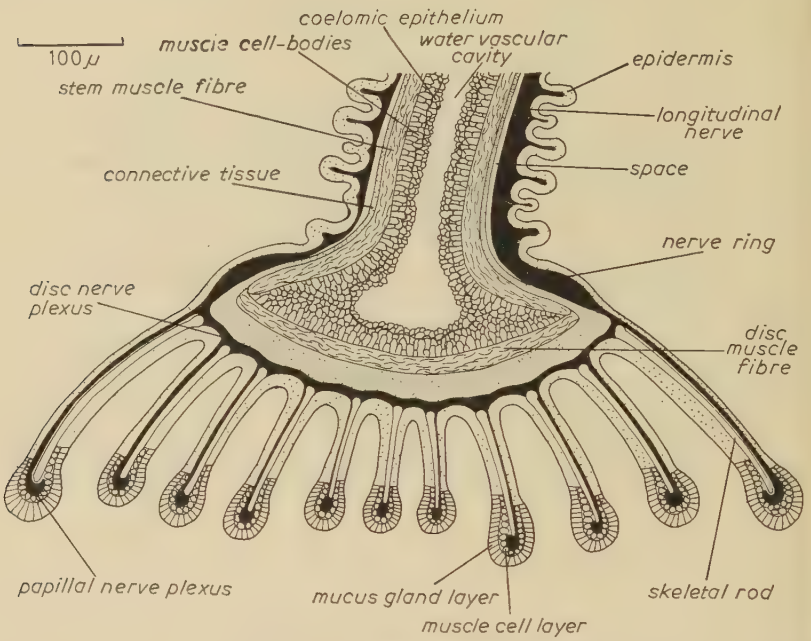


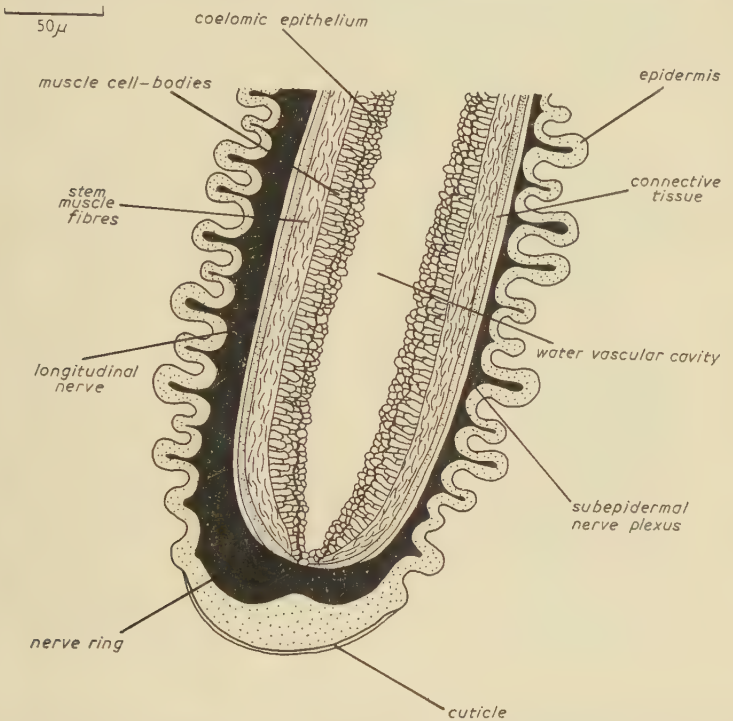
Fig. 8. Diagram of a L.S. through a sub-anal tube-foot, passing through the longitudina tract on the right side of the stem and 11 of the 100 or so papillae which arise from the disc There is a specialized mucus-secreting epithelium at the tip of each papilla.

side of the disc nerve plexus. The significance of these differences is discussed below.

As in the oral tube-feet, the mucus-secreting epithelium is confined to the knobs at the distal ends of the papillae, but the epithelium is more like that of the disc and papillae of the dorsal funnel-building tube-feet. Apart from aggregations of melanin, the outer layer of the epithelium is almost entirely composed of single-celled mucus-glands, 16–20 μ long and 2–5 μ wide, which can best be shown by staining in mucicarmine; their nuclei are in general long and thin and fairly dense. Between the mucus-glands are muscle-fibres about 1 μ thick, three or four usually being associated with each gland. Their cell-bodies, internal to the layer of mucus-glands, have round nuclei, 3–4 μ in diameter, which are less dense than those of the mucus glands. Sensory

ocesses with small oval nuclei associated with them, arise from this ithelium.

Serial sections through one such tube-foot showed a distinct region ecialized for mucus-secretion inside the fringe of papillae very like the ecialized disc of the funnel-building tube-feet. This region contained ucus-glands and muscle-fibres, and was clearly fully developed for burrow-ilding. The fringe contained about 50 papillae in 4 rows, each with a



G. 9. Diagram of a L.S. through a sensory tube-foot, showing the longitudinal nerve tract (on the left) and the concentration of nervous tissue at the distal end.

rminal epithelium closely similar to that of the disc. The facts suggest that e specialized disc in this case was an incipient papilla which had not yet veloped a stalk with its central skeletal rod. During the development of the be-foot, therefore, it seems likely that the specialized epithelium of each pilla is laid down first over the surface of the disc, and this is later pushed t by the development of a stalk. In this way the whole of the disc becomes vered by a velvet-like pile of papillae.

The Sensory Tube-feet

The only notable features of the histology (fig. 9) which may be said characterize these tube-feet are the absence of a disc as such and the gh proportion of nervous tissue. As in the stems of other tube-feet, a

subepidermal nerve plexus is present which is thicker on that side nearest the midline of the ambulacrum (left-hand side of fig. 9). At the distal end of the tube-foot the plexus is very much enlarged into a region homologous with the annular plexus of other tube-feet, though in this case, in all the specimens I have examined, the swelling is greatest on the side of the longitudinal nerve tract. I have not found any mucus-glands in these tube-feet, but the epithelium of the distal end is thicker than elsewhere with connective tissue fibres (most likely homologous with the 'supporting fibres' of Smith, 1937) traversing it from the nerve plexus to the outside. At the distal end the epithelium is bounded by a fairly thick cuticle. I have not found any cilia, similar to those in the disc and papillae of the dorsal funnel-building, sub-anal, and feeding tube-feet, in these tube-feet. Their structure suggests that the mode of operation of these tube-feet is as follows: As the urchin burrows these are extended slightly and touch the particles of the substratum with their heavily cuticularized distal ends. The tactile pressure is then relayed to the concentration of sub-epidermal nervous tissue by the connective tissue ('supporting') fibres lying in the epidermis and thence down the longitudinal nerve tracts to the radial nerves in each ambulacrum.

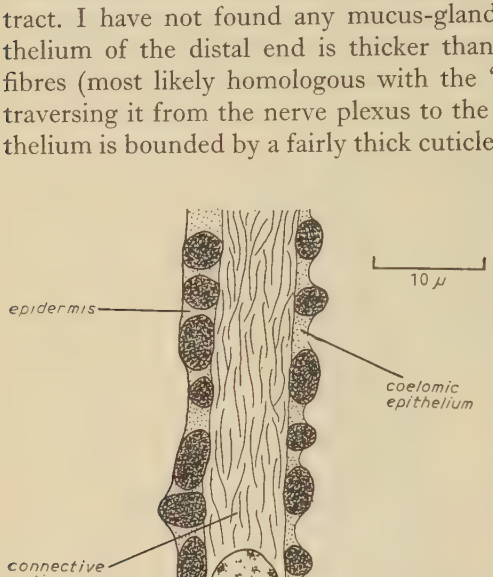


Fig. 10. Drawing of a L.S. through part of the wall of a respiratory tube-foot.

The Respiratory Tube-feet

The wall (fig. 10) is about 10 μ thick, and consists of three layers:

1. The outer epidermis is heavily nucleated, the density of nuclei being about one every 2–3 μ. The nuclei are some 2–3 μ in diameter, and consequently in places they appear to touch each other.

2. Internal to the epidermis is a diffuse connective tissue about 5 to 6 μ thick, consisting of longitudinally-running fibres in which aggregations of melanin (especially distally) and coelomocytes may be embedded. The nuclei are larger than the others in these tube-feet, reaching about 5 μ diameter in places; in fixed preparations these nuclei have very diffuse chromatin with a large and conspicuous nucleolus.

3. The coelomic epithelium in these tube-feet is quite well marked; the cells are less densely placed than in the epidermis (one every 4–5 μ), though the nuclei are very similar in size and appearance. The cytoplasm, however, is somewhat thinner.

The Clavulae of the Sub-anal Fasciole

The stem. In transverse section the stem (fig. 5, D) is round or slightly
al, about 50 μ in diameter. On two sides, diametrically opposite each other,
bands of cilia, always arranged with the cilia pointing along the line of the
ciole and perpendicular to the thorny processes of the calcite rod (Gordon,
26, p. 297 and fig. 26*f*). These bands are approximately 20 cells wide, but,
can be seen from fig. 5, D, their nuclei occupy much wider crescent-shaped
as between the central skeletal rod and the periphery, the cells converging
wards their free borders so that their cilia emerge along a band on each
le, some 10–20 μ wide. The nuclei of these cells stain uniformly and in-
sely black with Hansen's trioxyhaematein, and are easily distinguished
m the epidermal nuclei, the chromatin of which breaks up to a dark-brown
iculum. Large aggregations of melanin occupy the peripheral regions be-
een the ends of the groups of ciliated cells, and also the central cavity to
me extent, lying in the interstices of the calcite skeleton. The cilia themselves
15–20 μ long.

The terminal knobs. These may reach a diameter of 100 μ, the epidermal
er being about 15 μ in maximum thickness and surrounding a central
rtion occupied by the expanded part of the calcite rod. A few nuclei lie
this region, and a network of connective tissue-fibres permeates the skele-
n. Surrounding the skeleton and underlying the epidermal layer is a layer
connective tissue, sometimes reaching 5 μ in thickness.

The epidermis is strengthened throughout by connective tissue-fibres and
ntains many single-celled mucus-glands. These are about 20 μ long and
me 5 μ wide, and the nuclei are round or oval, normally 3 μ in diameter.
gregations of melanin occupy large regions of the ectoderm, which may
come swollen to 20 or 30 μ by their presence. I have not detected any
rvous-tissue in the knobs.

Discussion

The respiratory and sensory tube-feet, clearly specialized in gross and
nute structure for the job they perform, are each unique. The burrow-
ilding (dorsal and sub-anal) and feeding tube-feet, however, have super-
ial similarities, chiefly their penicillate structure, which closer examination
ntradicts. The chief points on which they differ, and which will now be
cussed, are the arrangement of the papillae, the position of the skeletal
ls, and the nature of the mucus-secreting epithelium.

The arrangement of the papillae. Since the mucus-secreting epithelium is
tricted to the disc and insides only of the papillae (except at the tip) in the
rsal funnel-building tube-feet, it is probable that mucus is plastered on to
funnel walls only during extension, and that as the tube-foot is with-
awn the papillae fold across the disc to minimize damage to the wall
ichols, in press). Observations in the present paper show that it is likely
t the folding over is brought about by the distal part of the stem muscle-
res, which terminate at the skeletal rods of the papillae. A similar though

less complete folding occurs during retraction of the sub-anal tube-feet though here the stem muscle-fibres do not terminate at the skeletal rod (fig. 8), and the disc centre is not free of papillae but contains smaller ones than at the fringe. It is unlikely that as much closure of this fringe is necessary in the sub-anal tube-feet, since the tube they maintain is not excavated from scratch, as is the narrower respiratory funnel, but remain as the sand falls in behind the burrowing animal, being originally moulded to shape by the sub-anal tuft of spines and merely maintained by the extending tube-feet. It is not surprising that the feeding tube-foot has no facility for papillal folding as this would be disadvantageous for its purpose. Hence, the disc is crowded with papillae, the tips of which form an almost flat sticky surface for picking up sand particles.

The position of the skeletal rods. In view of the different powers of articulation in the papillae, it is not surprising that the proximal parts of the skeletal rods are differently embedded. Those of the funnel-building tube-feet, which are concerned in the mechanics of papillal folding, and those of the sub-anal tube-feet, with papillae that fold passively during retraction, are not embedded in the substance of the disc, whereas the proximal ends of the feeding tube-feet lie deep in the disc connective tissue to ensure rigidity.

The mucus-secreting epithelium. The presence of the special muscle-operated mucus-glands of the discs and papillae of the burrow-building tube-feet can be explained as a device for more efficient discharge of mucus in those cases where the mucus needs to be shot out and plastered on to the walls of the burrow, not retained on the tube-feet. The converse is true for the feeding tube-feet; here a sticky area is required for picking up sand, and no muscle-operated glands occur.

The phenomenon of muscle-operated mucus glands, reported here for the first time in echinoderms, is by no means unique. Fretter and Graham (1949, 1954) report several instances in their work on the histology of the mantle-edge of gastropods such as *Odostomia* (1949, p. 495 and fig. 2) and *Siphonaria* (1954, p. 578 and fig. 8). Graham also remarks (1957, p. 135) that muscle-operated mucus glands occur round the mantle edge of such gastropods as *Calyptraea*, *Crepidula*, and *Onchidella*, and Morton (1955, p. 177) describes narrow strips of muscle round the glands of the foot in *Otina otis*. The most striking convergence, however, is provided by the edge of the mantle skirt of *Patelloida virginea* (Fretter and Graham, 1954, p. 579 and fig. 9) in which the innermost layer of glands is surrounded by a basket-work of muscle-fibres and between which permeate a great plexus of nerves. In addition the epithelium contains long, motionless cilia which these authors suggest may be sensory fibres relaying local stimuli by way of the plexus to the muscle-cells, the mucus which they help to secrete being toxic to small animals.

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The Embryology of the Polychaete *Scoloplos armiger*

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Summary

The eggs of *Scoloplos armiger*, laid in gelatinous cocoons, are yolky. Spiral cleavage and gastrulation produce a yolky trochophore, which then develops 12 (embryonic) trunk segments in strict succession. Ciliated bands occur, though development is non-larvagic. Segment-formation ceases after 8 days and the embryo hatches and metamorphoses into a feeding pre-adult, cilia being lost and yolk resorbed. Further (post-embryonic) segments are now added to the trunk. Anterior to the first trunk segment is the prostomium and mouth region, derived from the main body of the trochophore. Posterior to the trunk segments is a prepygidial growth zone, followed by a terminal pygidium.

The 12 embryonic trunk segments each contain a pair of hollow mesodermal somites descended from 4d, with ectoderm derived from 2d. The endoderm, of 3A–3C and 4d origin, meets the stomodaeum anteriorly and the pygidial ectoderm posteriorly.

The prostomium arises from part of the apical plate, derived from 1a–1d, and includes the cerebral ganglion. The ectoderm of the mouth region, partly apical plate, partly 2d in origin, includes transitorily the prototroch cells. Stomodaeum and proboscis apparatus develop in the mouth region from second and third quartette cells. The stomodaeum forms the pharynx and part of the oesophagus.

An anterior median and three pairs of lateral coelomic cavities arise in the 4d mesodermal bands in the prototrochal region (prostomium and mouth region), soon becoming confluent with each other and with those of the first two trunk segments as the adult head coelom.

The ectoderm of the embryonic segments forms epithelium, chaetal sacs, and ventral nerve ganglia. The endoderm forms oesophagus, stomach, intestine, and rectum. The somites give rise to peritoneum, trunk musculature, and blood-vessels.

The post-embryonic segments have a similar origin and development to the embryonic. The mouth region and first trunk segment fuse to form the peristomium. The pygidium, a 2d ectodermal structure, undergoes little change.

A trochophore stage occurs in development in all polychaetes, variously modified according to size and yolkiness of the egg. The simple planktotrophic condition appears to be primitive. The trochophore body comprises a prototrochal region, growth zone (giving trunk segments) and pygidium.

In polychaete trunk segments, 2d ectoderm forms epithelium, chaetal sacs, and ventral ganglia. Each segment contains a pair of hollow mesodermal somites, usually 4d origin, segmenting before the ectoderm. In Nereidae and Serpulidae are found specializations associated with heteronomy of the segments, a secondary phenomenon in polychaetes. The walls of the somites form peritoneum, trunk musculature, and blood-vessels. The haemocoel corresponds to the blastocoel.

The polychaete gut arises from stomodaeal and endodermal rudiments. In yolky forms differentiation is delayed. Proboscis development differs in different families.

The polychaete pygidium is an ectodermal post-segmental structure bearing the anus.

The polychaete head is variable and specialized. Prostomium and mouth region, derived from the prototrochal region of the trochophore, are probably presegmental. The mouth region usually combines with the first trunk segment to form the

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peristomium. No direct comparison can be made of the polychaete with the onych phoran-arthropod head.

INTRODUCTION

RECENT investigations of polychaete development have been concern almost exclusively with external changes (see Thorson, 1946; D. Wilson, 1948 for summaries), and apart from the work of D. P. Wilson (193 on the highly aberrant *Owenia fusiformis*, little attempt has been made duri the past 40 years to examine in any detail the internal epigenetic process which underlie them. As a consequence, our knowledge of polychaete ep genesis is extremely limited. Based on scattered papers published main before 1900, it is fragmentary, contradictory, and inconclusive, and in no ca provides a complete account of the embryology of any one species from e to adult. Cleavage and the development of the trochophore are reasonably w understood (von Wistinghausen, 1891; E. B. Wilson, 1892, 1898; Mead, 189 Eisig, 1898; Child, 1900; Treadwell, 1901; Delsman, 1916), but the later stag leading to the formation of the adult are much less clear, so that for compar tive purposes little reliable information is available. Ectoderm, endoderm, a

eral kinds of mesoderm have been recognized, but the final contribution ich they make to the structure of the adult requires detailed elucidation. The paucity of information on this subject has led to considerable delay in solution of certain problems of comparative morphology. Of latter years, ch has been written concerning the segmental composition of the trunk in mented invertebrates, and as a result the theory of Iwanoff (1928, 1944) t the trunk in all segmented animals can be divided into anterior and sterior regions in which the segments differ in origin and development eory of primary and secondary segments) has been largely refuted (see ecially Manton, 1949). Iwanoff applied his theory to polychaetes as well to other animals, however, and lack of embryological evidence has so far vented a critical reappraisal of his views for this group.

A further recent controversy concerns the composition of the head in segnted invertebrates (Manton, 1949; Raw, 1949; Ferris, 1953; Hedgepeth, 54; &c.). Rival theories, based on morphological and embryological evince respectively, make assumptions about the segmental nature of the ychaete head which can neither be confirmed nor denied on the basis of sting authority. Once again, only epigenetic studies can provide the answer. For these reasons, therefore, the present investigation has been pursued, king information on the development of the adult tissues, the segmental mposition of the trunk, and the composition of the head, from the developntal stages of the little-known polychaete *Scoloplos armiger*.

The Pattern of Reproduction in *Scoloplos armiger*

S. armiger O. F. Müller (fam. Ariciidae) is one of the commonest polyetes burrowing in littoral and sub-littoral muddy sand. The adults, cribed by Mau (1882) and Eisig (1914), occur in large numbers in the dy Flats at Whitstable, on the north Kent coast. Newell (1954) gives the nsity of the species in this locality as about 40 per square metre, males and hales being found in approximately equal numbers. In June, when gamete liferation begins, no sexual difference can be seen externally, but by tober the accumulated genital products in the coelom impart a white oration to the males and a brown to the females (Mau, 1882). The gonads situated in the abdominal segments of the middle two-thirds of the body. the ripe female these segments are also characterized by white swellings he bases of the parapodia, formed by the glandular walls of the segmental hridial ducts (Mau, 1882).

n European waters spawning takes place in the spring. At Cuxhaven it urs in March (Schultze, 1855), off Holland from the end of February to middle of April (Ritzema Bos, 1874; Horst, 1880; Groot, 1907; Delsman, 6), off Denmark from March to May (Leschke, 1903; Thamdrup, 1935; nke, 1939; Thorson, 1946; Smidt, 1951), off Scotland in February (Cunigham and Ramage, 1888) and at the Isle of Man from March to April ornell, 1891). At Whitstable it has been observed to occur in two successive sons in March.

In both males and females the ripe gametes are passed out through th nephridial ducts of the genital segments (Groot, 1907). The eggs are laid gelatinous cocoons, the jelly of which is secreted by the glandular nephridi ducts of the female. As pointed out by Thorson (1946), the occurrence of su cocoons implies some form of copulation or pseudocopulation. Groot (190 states that copulation occurs in *S. armiger*, but unfortunately it has not bee possible to make further observations on this point. The absence of copulato organs in the male worms and of sperm or fertilized eggs in the coelom of th females, however, suggests pseudocopulation as a more likely possibility.

The cocoons, described and figured by several authors (e.g. Linke, 193 are pear-shaped structures, variable in size, but typically about 2 cm lo by 1 cm broad, and are anchored in the sand each by a gelatinous stalk son 5 cm long. The cocoon itself lies on the surface of the sand. It contains fro 600 to 1,800 eggs (Smidt, 1951) and appears pink, although the jelly itself colourless. The surface of the cocoon is covered by a layer of small adhere sand-grains. Since a ripe female may contain between 3,000 and 5,000 egg several cocoons must be produced by each female (Thorson, 1946). Althou the process of cocoon formation has never been observed, support for th idea is provided by the fact that on the beach cocoons are found in groups two to four, each group no doubt being produced by a single female.

Owing to the fecundity and high population density of *S. armiger*, cocoo are produced in vast numbers. The impression of this is enhanced by th occurrence, not previously recorded, of a main spawning which involv almost the whole of a littoral population and is related to a spring tide con bined with a rise in temperature. At low tide following such a spawning th entire area of the Whitstable Flats is covered by cocoons in a density approac ing 100 per square metre. Similar phenomena occur in other polychaet (Dales, 1950), but nothing is known of the causal mechanisms involved.

Early development in *S. armiger* takes place within the jelly of the cocoo and the embryos hatch (at Whitstable) in 13 to 15 days. They immediate burrow in the sand. Leschke (1903) reports hatching at the same stage at Ki in 21 to 24 days, while under laboratory conditions at 15° C it has been foun to occur in 10 days. The discrepancy in hatching time at Whitstable and Ki may therefore be a reflection of a general temperature difference between th two habitats during the breeding months.

Material and Methods

The pattern of reproduction in *S. armiger* greatly facilitates the collectio of material for embryological purposes. Large numbers of cocoons can b obtained very easily, all containing embryos at approximately the same stag of development. Cocoons were gathered during the breeding seasons of 195 and 1955 and maintained in bowls of clean sea-water at 15° C. Whilst in th cocoons the embryos fed entirely on yolk-reserves and the cultures require little attention. After hatching, the young worms were transferred to bow of sea-water containing a little of the muddy sand from the natural habitat o

animal. They proved to be very hardy and lived and grew in culture for ong as was required, feeding at this stage on diatoms in the sand. No ificant difference was observed between artificially reared worms and se collected directly from the beach.

f the fixatives employed in the preparation of serial sections, Smith's naldehyde dichromate was found to be the best for the pre-hatching stages aqueous Bouin the most useful for the older stages. Sections were em-ded in paraffin (m.p. 52° C), cut at 8μ, and stained with Delafield's matoxylin and eosin and, in the case of the later stages, with Heidenhain's n triple stain. The use of Delafield's haematoxylin, unusual in this type ork, was dictated by the fact that the normally more precise Heidenhain's matoxylin and Heidenhain's Azan stains were strongly taken up by the reserves of the embryos, producing a uniformly obscure preparation. afield's left the yolk unstained. While intracellular details were not aled by such methods, cell boundaries and nuclei were clearly displayed. everal hundreds of embryos were examined during the course of the stigation, in the living state, as whole mounts, and in serial section. From accumulated data it has proved possible to reconstruct the course of geny as far as an advanced pre-adult stage. Both the development of the rnal features and the internal changes which accompany their formation described below. Particular attention is paid to the relationship between apparent segmental composition of the body, manifested externally, and true segmental structure revealed by the mesoderm. External changes are sidered first.

External Features of Development

embryonic period

he unfertilized egg of *S. armiger* is opaque, spherical, 250μ in diameter, nge by reflected light, brown by transmitted light, and covered by a trans-nt egg membrane 2μ thick. When fertilization occurs this membrane omes raised from the surface of the egg and two small polar bodies appear he intervening space. Spiral cleavage and gastrulation follow (see Delsman, 6).

y the end of cleavage the embryo has become a spherical blastula pressing tly against the fertilization membrane, which persists as the embryonic cle. The opacity of the blastula, due to yolk globules in all the cells, cures the course of gastrulation, but in strong transmitted light the tocoel can be seen to have disappeared by the end of the second day. The ryo now begins to increase in length and prototrochal cilia appear, pro-ng through the cuticle. In the early 3-day embryo of fig. 1, B the proto-h (*p*) is well developed. Its dorsal and mid-ventral gaps persist throughout elopment.

uring the third day a pair of red eyespots (*e*) appears dorso-laterally at the rior margin of the prototroch. Increase in length continues and the rnal form of the embryo begins to change. In the embryo of fig. 1, C a

broad pointed anterior prototrochal region is demarcated by lateral groov from a narrower trunk. At the anterior end of the trunk a further pair grooves marks the limits of the metatrochal or first trunk segment, whi bears the lateral rudiments of the metatroch (*me*). The embryo now mov through the jelly of the cocoon by ciliary gliding, but shows no muscul activity.

On the fourth day the neurotroch (*n*) and the telotroch (*t*) appear. The fir is a mid-ventral band of short cilia, broad anteriorly, extending from th anterior margin of the metatrochal segment to a point just short of the tel troch. The telotroch originates, like the metatroch, as a pair of lateral ban of short cilia, but soon extends dorsally and ventrally to form a transverse ri with a small mid-ventral gap. When complete, it separates the termin pygidium from the growing trunk (fig. 1, D, E). The pygidium undergoes 1 further external differentiation until the eighth day.

From the fourth to the eighth day new material is continually added to th trunk from the growth zone immediately anterior to the telotroch, and at th same time segments are progressively demarcated behind the metatroch segment (figs. 1, C–I; 2; 3, A, B). The rate of growth of the trunk and the ra of differentiation of the segments are such that an undifferentiated zone som 150μ in length is always present at the posterior end of the trunk through the period.

By the end of the fourth day the metatroch is complete and the embr shows lateral muscular flexion of the prototrochal region and metatroch segment. Ciliary gliding continues, and becomes more marked as the cil of the various bands increase in length. During the fifth day a further narro ring of cilia with a small mid-ventral gap, the akrotroch, appears just anteri to the prototroch (*ak*, fig. 1, D, E), the second trunk segment is delineated an muscular activity becomes more obvious and more complex. In addition lateral flexion of the anterior end, the region of the embryo between th posterior border of the akrotroch and the anterior border of the metatroch segment undergoes successive lengthening and narrowing followed by shorte ing and widening. Such activity is the basis of peristaltic movement an indicates the presence of both circular and longitudinal contractile element but is not yet sufficiently co-ordinated to result in forward movement.

The anterior tip of the prototrochal region is periodically retracted, formi a small pit.

The length of the embryo continues to increase, mainly by addition to th trunk but partly by growth of the region between the posterior border of th

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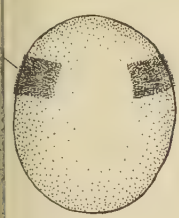
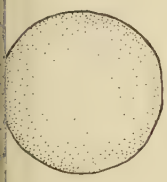
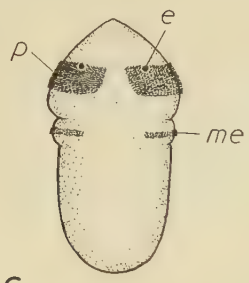
FIG. 1. A, unfertilized egg. B, early 3-day embryo, dorsal view. C, early 4-day embry dorsal view. D, late 5-day embryo, dorsal view. E, late 5-day embryo, ventral view. F, la 6-day embryo, dorsal view. G, late 6-day embryo, lateral view. H, early 7-day embryo, dors ew. I, early 7-day embryo, ventral view.

ak, akrotroch; *ann*, post-prostomial annulus; *ch 1*, *ch 2*, *ch 5*, chaetiger 1, 2, 5; *cil*, cili *e*, eye; *g 1*, *g 2*, *g 3*, gastrotrochs of chaetigers 1, 2, and 3; *m*, mouth; *me*, metatroch; *n*, neur troch; *p*, prototroch; *t*, telotroch.



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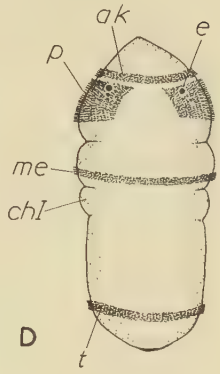
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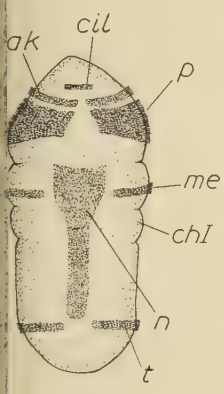
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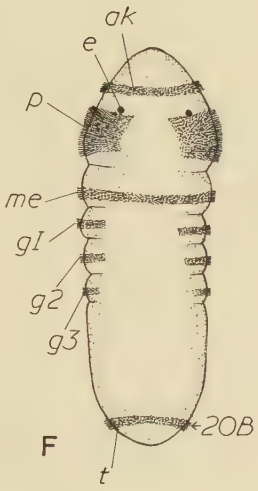
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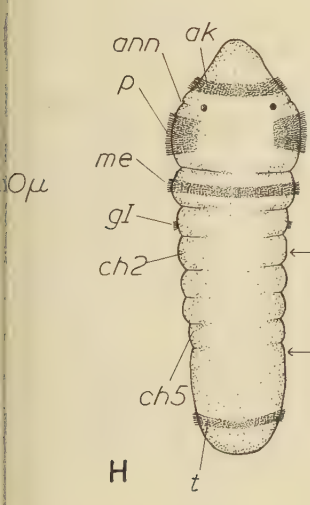
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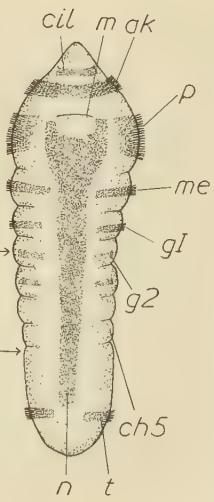
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akrotroch (*ak*) and the anterior border of the prototroch (*p*, fig. 1, F, G). front of the akrotroch a short transverse band of cilia (*cil*) appears mi ventrally, while more posteriorly, forward growth of the lower lip makes t mouth (*m*) conspicuous and carries the anterior end of the neurotroch forward across the prototrochal region. Two further segments are demarcat in the trunk, and each of these, as well as the second trunk segment, bears gastrotroch (*g 1*, *g 2*, *g 3*) with a mid-ventral gap. Muscular elongation a

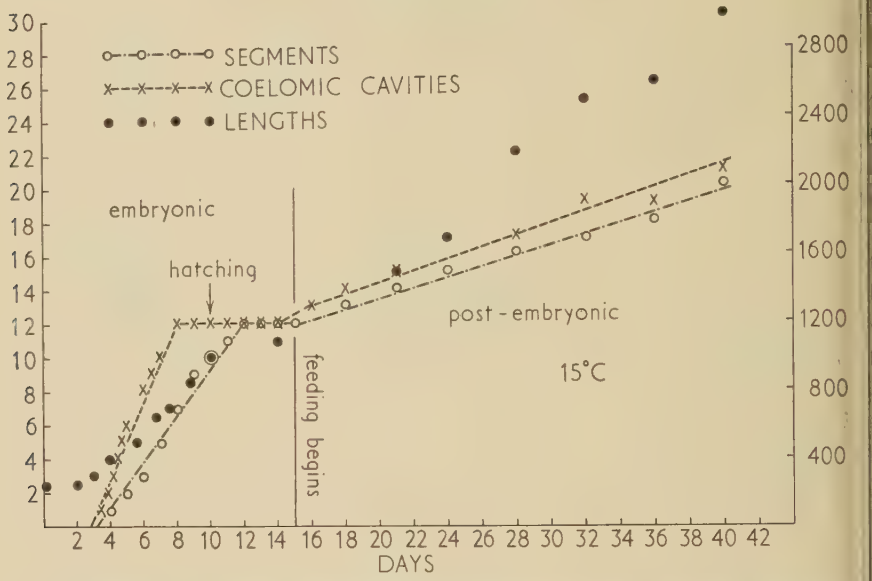


FIG. 2. Formation of paired coelomic cavities of trunk segments, external delineation trunk segments, and length of embryo and pre-adult in *S. armiger*, plotted against ti Ordinates: on the left, number of units; on the right, length.

contraction extend as far back as the posterior border of the metatroc segment.

On the seventh day the fifth and sixth trunk segments become marked and all intersegmental grooves become much more conspicuous (fig. 1, H, The cilia in the various bands attain a maximum length and ciliary glidi movements are very strong; but in spite of this the embryo is affected by t onset of metamorphosis. The prototroch (*p*) lessens in area, leaving a wi dorsal gap. The gastrotrochs, too, become shorter. In the prototrochal regi lateral grooves (*ann*) develop, marking off the anterior prostomium and leavi the prototroch on a distinct mouth region. Lateral flexion of the embryo a elongation and contraction spread back to the second trunk segment.

On the eighth day (fig. 3, A, B) two more trunk segments appear and t contractility of the embryo greatly increases. At the same time, althou movement of the embryo through the jelly is still by ciliary gliding with t anterior end extended, the various ciliated bands become much smaller. T

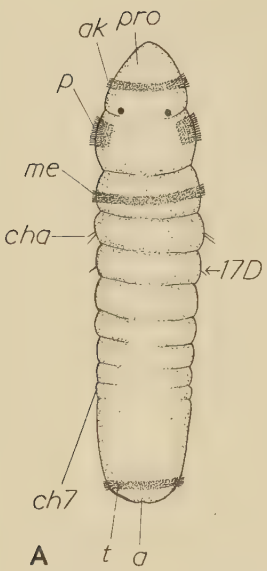
dual disappearance of the prototroch results in a decrease in size of the uth region, now strongly marked off from the prostomium (*pro*), while the stomium itself becomes more compact. The first chaetae (*cha*) appear as pairs on the second trunk segment (chaetiger 1) and one pair on the third aetiger 2), notopodial in both cases. The pygidium (*py*) begins its differenion with the formation of a small terminal proctodaeal pit (*a*). Muscular vement in the embryo is of the same type as hitherto, but extends over first 7 trunk segments and includes dorso-ventral as well as lateral flexion. s at this stage that the activity of the growth zone temporarily ceases. From ninth to the end of the fourteenth day the changes which take place are ted entirely to metamorphosis and general overall growth. In the unsegnted posterior region of the 8-day embryo the remaining embryonic ments gradually become defined (fig. 2). Once the twelfth trunk segment aetiger 11) has appeared on the twelfth day, no further segments are added il metamorphosis is complete.

Reduction of the various ciliated bands is well advanced by the ninth day, l with it a change occurs in the behaviour of the embryos in the jelly. iary gliding ceases, although the cilia that remain are still beating actively. the same time the spasmodic muscular peristalsis, now extending back to eighth trunk segment, works in conjunction with the externally projecting etae to produce occasional forward motion. Only one pair of chaetae ears on the ninth day, a notopodial pair on the fourth trunk segment aetiger 3).

On the tenth day all ciliated bands other than the prototroch (*p*), metatroch), and neurotroch disappear completely and those which remain become atly reduced in size (fig. 3, c). There is a rapid increase in the number of etae, and the locomotory peristalsis shows greater co-ordination and extends k as far as the ninth trunk segment (chaetiger 8). Although still interrsed with periods of random flexion it now results in definite, if irregular, ward progression. By this means the embryo leaves the cocoon and burrows the surrounding sand. All the viable embryos in a cocoon hatch within a hours. Burrowing is, in part at least, a negatively phototactic response pton, 1953). Newly hatched embryos in sand in a dish illuminated from ow will migrate to the surface of the sand.

Although after 10 days the embryos have a well-developed, co-ordinated uscular system, metamorphosis from the yolky condition to a fully differented feeding pre-adult is not completed until 5 days later. During these ays the protection previously afforded by the cocoon is lost and the mortal risk to the embryos therefore greatly increased. It may be significant in nnexion with this that the residue of unfertilized eggs and abnormal bryos in the cocoon, and the jelly of the cocoon itself, begin to putrefy the time that hatching occurs. This phenomenon has been observed both the laboratory and on the beach. The cocoon, in fact, appears to become inhabitable at this stage of embryonic life.

Throughout the period of metamorphosis the embryo continues to grow,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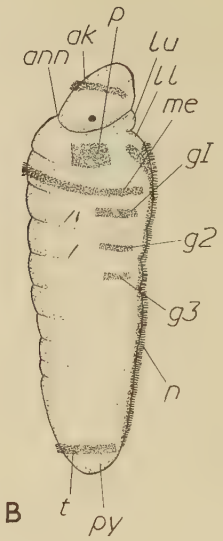
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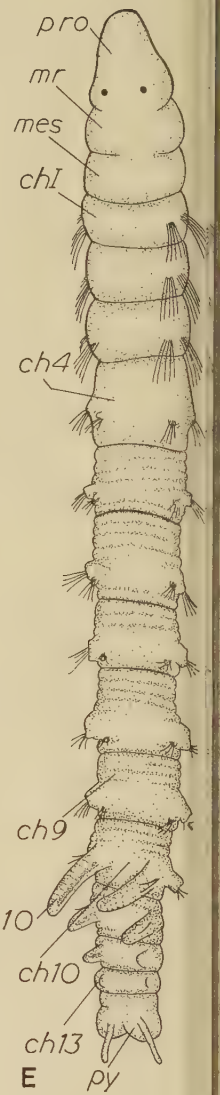
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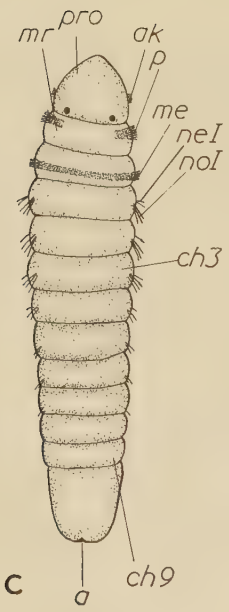
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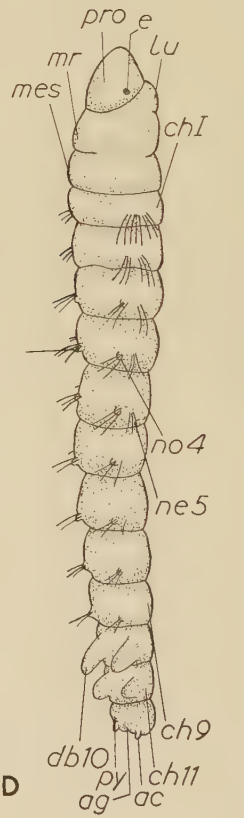
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ut more slowly than when the growth zone is active (fig. 2). The last two mbryonic segments become defined successively and each develops a pair of unded latero-dorsal protuberances, the rudiments of dorsal branchiae (*db 10*, *11*). Fig. 3, D illustrates the condition of the embryo towards the end of etamorphosis. The disappearance of the prototroch, together with all other liated bands, results in a decrease in size of the mouth region (*mr*). At the me time forward growth of the region ventrally, in association with the velopment of the mouth, displaces the prostomium antero-dorsally. The oboscis of the future pre-adult first becomes protrusible on the eleventh y. The metatrochal segment (*mes*) remains distinct and does not develop aetae. On the remaining embryonic trunk segments the chaetae continue develop evenly from before backwards. Those of chaetigers 1 to 3 form terally placed notopodial and neuropodial fans, while the notopodial chaetae the fourth (no. 4) and succeeding chaetigers are dorsally placed and the uropodial laterally placed. The notopodia of chaetigers 4 to 9 develop small otuberances, one pair to each segment, which will become the dorsal noto-dial cirri (*dc*). On the two segments bearing dorsal branchiae no chaetae pear until after the end of metamorphosis. The branchiae of chaetiger 10 (*b 10*) elongate considerably between the eleventh day, when they first appear, d the fifteenth. Those of chaetiger 11 do not begin to develop until the urteenth day, although the segment itself is delineated on the twelfth, and e still very small by the end of metamorphosis.

On the twelfth day a new element appears in the behaviour of the embryo ith the secretion of mucus from the body surface in quantities sufficient to nd the sand grains adjacent to the animal into a temporary tube. By the time etamorphosis is complete all 12 segments of the embryonic trunk act in -ordinated locomotory peristalsis. Chaetiger 10 attains this condition on the eventh day, but chaetiger 11, developing more slowly than the preceding gments, does not show muscular activity until the fourteenth day. In the wly metamorphosed pre-adult, locomotion is essentially of the adult type; is emphasizes the morphological differentiation between the three anterior aetigers and those posterior to them. The former are thoracic in structure d play a major part in burrowing. When a wave of circular muscle contrac-n passes from before backwards, the mouth region, metatrochal segment, d three anterior chaetigers successively elongate and the chaetae of the latter e flattened against the body-wall. Since the more posterior part of the body anchored by its projecting chaetae, the anterior end pushes forward through e sand. The subsequent wave of longitudinal muscle contraction shortens

FIG. 3. A, late 8-day embryo, dorsal view. B, late 8-day embryo, lateral view. C, 10-day bryo, dorsal view. D, 14-day embryo, dorsal view. E, 21-day pre-adult, dorsal view. *a*, anus; *ac*, anal cirrus; *ag*, anal groove; *ak*, akrotroch; *ann*, post-prostomial annulus; *ch 1*, *2*, &c., chaetiger 1, 2, &c.; *cha*, chaeta; *db 10*, dorsal branchia of chaetiger 10; *e*, eye; *g 1*, *g 3*, gastrotrochs of chaetigers 1, 2, 3; *ll*, lower lip; *lu*, upper lip; *me*, metatroch; *mes*, tatrochal segment; *mr*, mouth region; *n*, neurotroch; *ne 1*, *ne 5*, neuropodium of chaetiger 1, *no 1*, *no 4*, notopodium of chaetiger 1, 4; *p*, prototroch; *pro*, prostomium; *py*, pygidium; elotroch.

and widens the three anterior chaetigers, and their chaetae project laterall and act as anchors. Chaetiger 1 becomes fixed first and the others are draw up to it. The fourth and succeeding chaetigers tend to be extended by t shortening of the more anterior ones and then to contract strongly from befo backwards. The emphasis in them is on longitudinal muscle contractio Cyclic repetition of the process of pushing forward with the anterior end the body and then drawing the more posterior part up to it results in burrowin

In the early hatched stages the protrusible proboscis also plays a part burrowing. After forward extension of the anterior end, proboscis protrusio occurs and the proboscis itself becomes attached, evidently by some form sticky secretion, to the relatively large surrounding sand grains. When t anterior segments contract the proboscis is simultaneously retracted and t worm is pulled forward on to it. By the time metamorphosis is complet proboscis activity also results in the ingestion of food (diatoms from the su face of the sand grains). Proboscis protrusion later becomes entirely dissociate from locomotion.

During metamorphosis the pygidium undergoes marked differentiatio (*py*, fig. 3, D). The proctodaeal pit extends dorsally and ventrally as a groo (*ag*) and on each side of it the two halves of the pygidium increase in size. T increase being greater ventrally than dorsally, the anus migrates to a poster dorsal position. On each side of the anus an anal cirrus (*ac*) develops. T dormant prepygidial growth zone lies just behind the posterior border chaetiger 11 and is not distinguishable from the pygidium during met morphosis.

The post-embryonic period

On the fifteenth day the prepygidial growth zone again becomes activ adding new, post-embryonic segments to the posterior end of the trun Overall growth also continues, with the result that the pre-adult increas both in length and in bulk (figs. 3, E; 6, A, B). For the first 4 weeks of pos embryonic life the rate of increase in size is more or less constant (fig. 2 The rate at which new segments are formed is also constant, but differs fro that obtaining during the embryonic period (fig. 2). The first external indic tion of the formation of a segment is an increase in length of the prepygidi region. After 3 (later 4) days an annulus is formed between the poster border of the segment and the growth zone. The first post-embryonic se ment, chaetiger 12, is demarcated on the eighteenth day, the second on t twenty-first (*ch 13*, fig. 3, E), and so on. Each segment undergoes an initi period of rapid development and, after 4 days, dorsal branchiae are present an the segment is co-ordinated with the rest of the body as a locomotory uni The dorsal branchiae characterize the post-embryonic segments as abdomin from their inception. The rate of formation of segments is such that the ne is just being cut off from the prepygidial growth zone as the one anterior t it completes its initial development (figs. 3, E; 6, A, B). This condition co trasts with the mode of formation of the embryonic segments, in which t

diments of a large number of segments are laid down very quickly and the sterior half of the embryo is largely undifferentiated. The difference is ated to the habits of the developing animal during the embryonic and post-mbryonic periods. Once the pre-adult has begun to move about actively and feed, its efficiency is lessened by the presence of a large undifferentiated owing zone. In the embryo, on the other hand, the presence of undifferentiated segments at the posterior end is not a disadvantage, since both food and otection are readily available. Chaetiger 11 shows an intermediate condition that it is laid down as an embryonic segment and remains undifferentiated om the twelfth to the fourteenth day, but then undergoes the rapid differentiation characteristic of a post-embryonic segment.

After 28 days of development the locomotory movements are wholly of e adult type. The first three chaetigers show strong peristaltic burrowing tivity, while the remainder of the body undergoes longitudinal contraction llowed by slow relaxation as the anterior end moves forward. Protrusion of e proboscis is now associated entirely with feeding.

The changes in external form which take place during the first 4 weeks of e-adult life are characteristic for each of the five body regions which can w be distinguished (figs. 3, E; 6, A, B).

The head. Evidence is presented below in support of the view that the head *S. armiger* comprises the prostomium (*pro*), the mouth region (*mr*), and the etatrochal segment (*mes*). The distinction between the mouth region and etatrochal segment becomes less obvious as development proceeds. The rostomium increases in size and becomes pointed anteriorly. The two red yespots persist laterally near its base. The mouth (*m*), lying below and hind the prostomium, widens transversely and its lips (*lu*, *ll*) become more ominent. The metatrochal segment shows no especial change other than crease in size.

The thorax. The first three chaetigers are distinguished by form and function as components of a distinct thorax. They are all relatively short and wide, th laterally placed chaetae. The segments increase gradually in size, and e notopodial and neuropodial chaetae, which are all of the simple, serrate pe, become longer and more numerous (*no 2*, *ne 2*, fig. 6, A). Although the topodial chaetae appear first in development, there is a general tendency r the neuropodial chaetae to attain a greater number in due course.

The transition zone. Chaetigers 4 to 9 are not specialized for locomotion and t do not bear the dorsal branchiae characteristic of abdominal segments. In of them the notopodial chaetae project dorsally and the neuropodial dorsoerally (*no 9*, *ne 9*, fig. 6, A). The chaetae and the notopodial cirri (*dc*) adually elongate and the body-wall becomes produced into neuropodial ojections in all segments except chaetiger 4. The projections are small and erally placed in chaetiger 5, but those of successively posterior segments e more dorsally placed and grow larger. In this way chaetigers 4 to 9 form zone of morphological transition between the last thoracic segment (chaetir 3) and the first abdominal (chaetiger 10). On chaetiger 4 the neuropodia

resemble those of the thoracic segments and after 6 weeks bear 8 curve chaetae which function in the same way as the thoracic chaetae. The segmen also remains short and wide. Chaetiger 9, on the other hand, develops almo in the same manner as chaetiger 10, except for the absence of dorsal branchia At 6 weeks it is relatively long and is externally subdivided by annuli.

The chaetae of the transition segments show no peculiarities in develop ment. They are of the same serrate type and undergo gradual increase i length and number evenly from before backwards. As in the thoracic segmen the notopodial chaetae appear slightly before the neuropodial.

The abdomen. The abdominal segments increase in number from 2 a metamorphosis to 10 at the end of 6 weeks. Each one follows the same cours of development. After the initial 4-day period already described (p. 100) th segment is short and wide, with a pair of blunt dorso-lateral branchial rudi ments and no chaetae (*ch 13*, fig. 3, E). With increase in size, however, th segment becomes much longer than wide. Growth takes place mainly anteri to the position of the dorsal branchiae, so that they and their associated para podia remain at the posterior end of the segment. The segment also become subdivided by secondary annulations and during the powerful longitudina muscle contraction these become much more conspicuous.

The dorsal branchiae increase in length as the segment grows and 4 day after their first appearance a longitudinal row of cilia (*dbc*) develops on th median surface of each. The beating of the cilia produces a current of wate flowing posteriorly over the dorsal surface of the abdomen. The branchia become annulated, but do not show longitudinal contraction, their onl movement being a backward and forward waving.

The parapodia of the abdominal segments begin to develop about 10 day after each segment has been formed. A pair of notopodial chaetae grows ou dorsal-wards just lateral to the base of each branchia. During the next 4 day a small projection of the body-wall appears on each side lateral to the noto podial chaetae, forming the rudiment of the neuropodium. The neuropodiu develops into a triangular lateral expansion of the body-wall bearing a increasing number of chaetae at its apex. The notopodial chaetae also increas in number and a small dorsal cirrus grows out behind each group.

The pygidium. The prepygidial growth-zone retains its simple cylindric form, but behind it the pygidium (*py*) continues the differentiation begu during metamorphosis. Ventral growth predominates and by the twenty-firs day the anus is dorsal in position. The median posterior groove persists an the two halves of the pygidium gradually increase in size. The anal cirri (*ac* also grow longer.

Later development

After 6 weeks the pre-adult enters on a new phase of development. Th formation of abdominal segments by the prepygidial growth-zone continue throughout life and the segments can all be identified as post-embryonic. A the same time a gradual increase occurs in the number of thoracic segment

the transition zone moves progressively backwards along the animal. mination of the internal anatomy of older pre-adults and adults reveals sign of growth zone at the posterior end of the thorax. The increase in nber of the thoracic segments results from the transformation of existing t-thoracic segments. Posterior migration of the transition zone is due to fact that in changing into thoracic segments the more posterior chaetigers s through stages which resemble the transition zone segments. Even the ginal anterior abdominal segments eventually become thoracic in form, but be identified by the presence of vestigial dorsal branchiae. In the head, mouth region, and metatrochal segment eventually fuse to form the istomium, separating the prostomium from chaetiger 1.

The external changes which take place as *S. armiger* develops from an egg o a pre-adult can thus be summarized as follows:

The egg first gives rise to a yolky trochophore with a broad prototroch. As embryo begins to increase in length, an anterior prototrochal region, ned from the main body of the trochophore, is demarcated from a growing nk. In the trunk 12 (embryonic) segments are successively defined. The t remains achaetous but carries a metatroch (metatrochal segment). The hainder become chaetigerous (chaetigers 1 to 11), developing paired noto- lia and neuropodia. Chaetigers 1 to 3 also acquire gastrotrochs, but these ated bands, together with the metatroch, the prototroch, an akrotroch, a l-ventral neurotroch, and a telotroch, are lost soon after hatching occurs. e telotroch, while it persists, separates a segment-forming growth-zone m a terminal pygidium derived from the posterior end of the trochophore. As formation of the embryonic trunk segments proceeds, the prototrochal ion anterior to them becomes externally subdivided into a prostomium and nouth region, the latter separating the prostomium from the metatrochal st trunk) segment. The mouth opens on the mouth region, and the anus the pygidium, just before hatching.

At 10 days, when the embryo hatches from the cocoon, it has a well- ordinated locomotory system and is able to burrow; but it continues to sist on yolk reserves for a further 5 days. During this period metamor- osis to an actively feeding pre-adult is completed, but there is no further rease in segment number. The newly metamorphosed worm has a pro- mium, a mouth region, 12 trunk segments, and a pygidium. As soon as it ins to feed, however, by means of its protrusible proboscis, the prepygidial wth zone once more becomes active and further (post-embryonic) seg- nts are successively added to the trunk. Segment formation continues oughout life. Anteriorly the mouth region and metatrochal segment fuse form the peristomium, separating the prostomium from chaetiger 1. steriorly the pygidium persists behind the growth-zone.

n order to understand the morphological relationship between the trunk ments and the prostomium, peristomium, and pygidium of *S. armiger*, it essential to know how segmentation is established, what constitutes a

segment and how far the development of the three latter body units resembl or differs from that of a segment. Further, the significance of the formati of the trunk segments in two successive series cannot be fully assessed relation to Iwanoff's theory of primary and secondary segments until the modes of origin and development have been compared. Thus a preci analysis of the epigenesis of the species becomes imperative. The followi pages describe the results of such an analysis.

Epigenesis

The trochophore

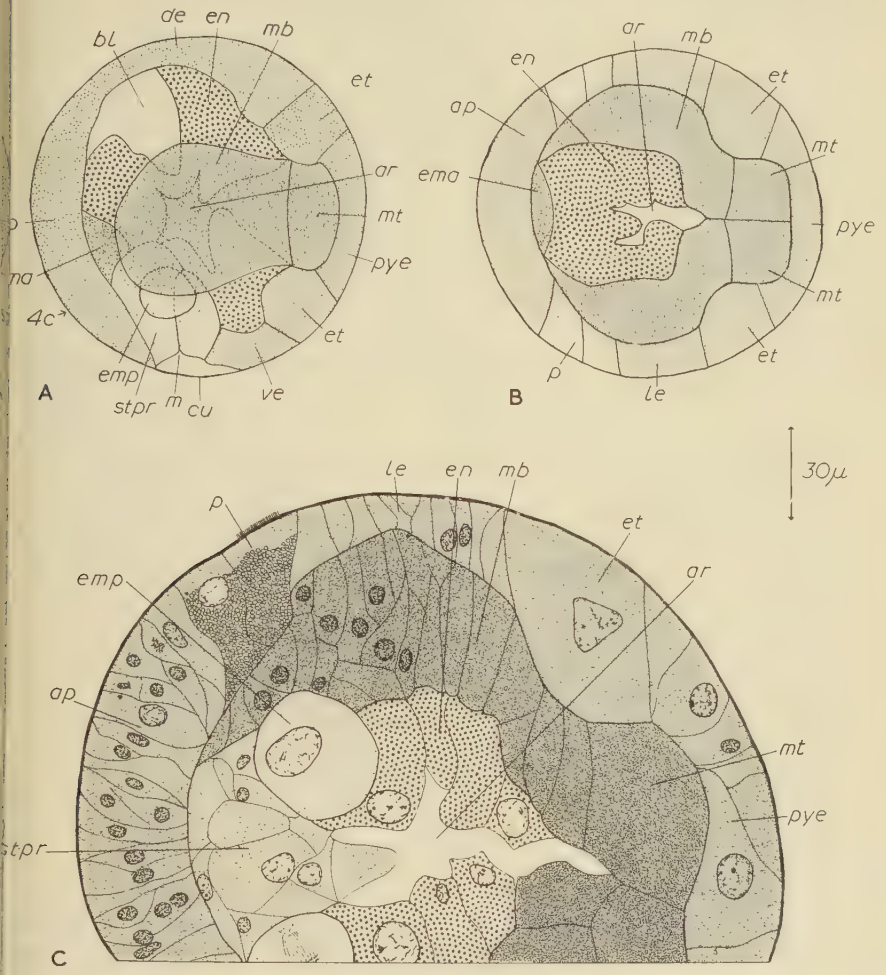
Towards the end of the second day the embryo of *S. armiger* attains th condition of a secondarily modified trochophore. As a result of the work Delsman (1916) it is possible to relate all its component parts to their orig in the cleavage blastomeres.

The form of the embryo is illustrated by fig. 4, A–C. Like the egg, it spherical and about 250μ in diameter. All the cells contain numerous yo globules (see prototroch cells (*p*) in fig. 4, C). At the anterior end of th embryo lies the apical plate (*ap*), derived from the animal pole descendan $1a^1$–$1d^1$ of the first quartette of micromeres. As a result of tangential an radial division the cells of the apical plate are already numerous and mo than one layer deep. Laterally the apical plate meets the prototroch cell These comprise 16 primary cells derived from $1a^2$ to $1d^2$ and two seconda cells $2a^1$ and $2c^1$. The primary cells are arranged in 4 groups of 4, two dors lateral and two ventro-lateral, and in each group two cells lie anteriorly an two posteriorly. The lateral gap in the primary prototroch row on each sid is filled by one of the secondary cells. The prototroch cells are the first differentiate and in fig. 4, C, are just becoming ciliated.

The small mid-ventral gap in the prototroch is filled by ectoderm derive from $2b^1$. Behind it lies the mouth (*m*). In the larger mid-dorsal gap the cel of the apical plate meet the ectoderm of the post-prototrochal half of th trochophore, all of which arises from the large blastomere 2d during gastrul tion and blastoporal closure. The 2d ectoderm consists of three distinct part Dorsally and laterally, and ventrally behind the mouth, it forms a single lay of relatively small cells (*de*, *ve*, fig. 4, A and *le*, fig. 4, B). Posterior to them li a transverse ring of 9 large cells, the teloblasts (*et*) of the ectoderm of th future trunk segments, and covering the posterior end of the embryo is further cap of cells, the rudiment of the pygidial ectoderm (*pye*). The anu is not present at this stage. Between the ventral ends of the ectoteloblast rin lie the teloblasts of the future neurotroch, the cells $3c^2$ and $3d^2$.

The mouth is formed from the anterior end of the blastopore, which nev closes completely. Associated with it is an ingrowing group of cells derive from the second quartette cells $2a^2$–$2c^2$ and the third quartette cells $3a^2$ an $3b^2$. These cells (*stpr*, fig. 3, A) later give rise to the stomodaeum and als contribute to the proboscis apparatus (p. 116). They meet the endoderm (*en* of the trochophore both dorsally and posteriorly. The endoderm, consistin

large cells descended from 4a to 4c of the fourth quartette and from the idual macromeres 4A to 4D, is by now arranged as a single layer around a ntral archenteron (*ar*). It makes contact with the ectoderm dorsally, ven-



. 4. A, late 2-day embryo, partially reconstructed in the sagittal plane. B, diagrammatic ntal section of embryo of fig. 4, A. C, frontal section of embryo of fig. 4, A at level indicated. apical plate; *ar*, archenteron; *bl*, blastocoel; *cu*, cuticle; *de*, dorsal ectoderm; *ema*, anterior omesoderm; *emp*, posterior ectomesoderm; *en*, endoderm; *et*, ectoteloblast; *le*, lateral oderm; *m*, mouth; *mb*, mesodermal band; *mt*, mesodermal teloblast; *p*, prototroch; *pye*, gidial ectoderm; *stpr*, rudiment of stomodaeum and proboscis apparatus; *ve*, ventral ectoderm.

lly, and anteriorly above the rudiment of the stomodaeum and proboscis paratus. Antero-dorsally part of the blastocoel (*bl*) still persists.

At the posterior end of the embryo the endoderm (*en*) is separated from the gidial ectoderm (*pye*) by the two large paramedian mesodermal teloblasts *t*) derived from 4d, and the ends of the solid mesodermal bands (*mb*) to

which they give rise. The mesodermal bands pass forward laterally between the endoderm and ectoderm as far as the prototroch. On each side of the rudiment *stpr* they run lateral to two large rounded cells (*emp*, fig. 4, C) descended from $3c^1$ and $3d^1$ and called by Delsman the posterior ectomesoderm cells or *Ektoteloblasten*. According to Delsman $3c^1$ and $3d^1$ give off during migration to their definitive positions, a number of small cells which come to lie between the mesodermal bands and the ectoderm. It has not proved possible to trace these cells in the present study, but they appear to be relatively unimportant to later development. The two posterior ectomesoderm cells (*emp*) form the major part of the rudiment of the proboscis apparatus (p. 116). Finally, at the anterior end of the embryo, between the apical plate (*ap*), the endoderm, and the rudiment *stpr*, lies a group of cells descended from $3a^1$ and $3b^1$ and called by Delsman the anterior ectomesoderm (*ema*).

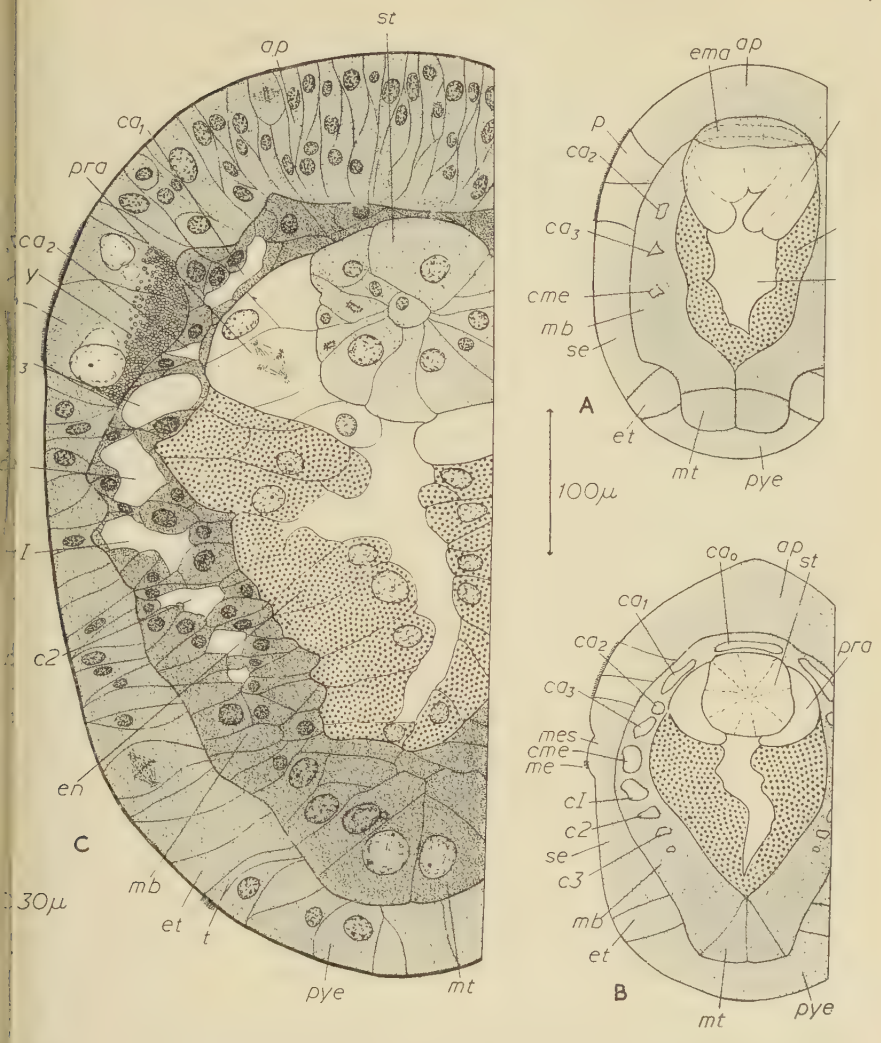
The cleavage products in *S. armiger* and their descendants in the trochophore may thus be summarized as follows:

$1a^1$–$1d^1$	apical plate ectoderm
$1a^2$–$1d^2$	16 primary prototroch cells
$2a^1$, $2c^1$	2 secondary prototroch cells
$2b^1$	pre-oral ventral ectoderm
2d	post-oral ectoderm, ectoteloblast ring, pygidial ectoderm
$2a^2$–$2c^2$, $3a^2$, $3b^2$	stomodaeum and proboscis apparatus
$3a^1$, $3b^1$	anterior ectomesoderm
$3c^1$, $3d^1$	posterior ectomesoderm (proboscis apparatus)
$3c^2$, $3d^2$	neurotroch teloblasts
4d	mesodermal teloblasts (mesodermal bands)
4a–4c, 4A–4D	endoderm

Formation of the embryonic trunk

The embryonic trunk comprises the 12 segments (metatrochal to chaetiger 11) laid down evenly from before backwards before metamorphosis (p. 97).

Ectoderm. The ectoderm of each segment is formed as a single layer of cells budded off anteriorly from the 2d ectoteloblast ring of the trochophore (p. 104) (*et*, figs. 4, A, B; 5, A, B; 6, C, D; 7, A, D; 8, A). The ectoteloblasts become active after the completion of blastoporal closure. Their earliest products form the ectoderm of the metatrochal segment (*se*, fig. 5, A) and later products the ectoderm of succeeding segments (figs. 5, A–C; 6, D, E; 7, A–D; 8, A; 9, A). The ectoteloblasts are larger ventrally than dorsally and this difference is emphasized in their daughter cells, the dorsal and dorso-lateral ectoderm being much thinner than the ventral and ventro-lateral (figs. 9, B–D; 10). Neurotroch cells are also budded off from the neurotroch teloblasts (p. 104) (*nt*, fig. 10) to form a mid-ventral band between the ventral borders of the segmental



5. A, late 3-day embryo, diagrammatic frontal section. B, diagrammatic frontal section of embryo of fig. 1, C. C, frontal section of embryo of fig. 1, C.
apical plate; *c 1*, *c 2*, *c 3*, coelomic cavities of chaetigers 1, 2, and 3; ca_0, anterior median omic cavity; ca_1, ca_2, ca_3, paired lateral coelomic cavities of prototrochal region; *cme*, omic cavity of metatrochal segment; *ema*, anterior ectomesoderm; *en*, endoderm; *et*, teloblast; *mb*, mesodermal band; *me*, metatroch; *mes*, metatrochal segment; *mt*, meso- nal teloblast; *p*, prototroch; *pra*, proboscis apparatus; *pye*, pygidial ectoderm; *se*, segmental ectoderm; *st*, stomodaeum; *t*, telotroch; *y*, yolk globules.

oderm (*nc*, figs. 9, B; 10). In their undifferentiated state the ectoderm and rotroch cells contain a considerable amount of yolk.

The ectoteloblasts gradually become smaller (*et*, figs. 4, C; 5, C; 7, B, C; A) and soon begin to divide perpendicular as well as parallel to the longi- dinal axis of the embryo, so that the number of cells in the ectoteloblast

ring increases. The ring remains identifiable, however (*et*, fig. 7, B), lyi just anterior to the telotroch (*tc*), and its cells continue to function as tel blasts. The ectoderm of the twelfth embryonic trunk segment (chaetiger 1 is laid down on the eighth day and the ectoteloblast ring subsequently persis unchanged throughout metamorphosis (*et*, figs. 7, C; 9, A). Its cells are larg than the differentiating ectoderm cells anterior and posterior to them.

Mesoderm and endoderm. In the trochophore the two 4d mesodermal tel blasts (*mt*) lie mainly within the pygidium, posterior to the level of the ect teloblasts (*et*, fig. 4, C), and the mesodermal bands (*mb*) are well develope before the ectoteloblasts become active. Consequently the mesoderm of th metatrochal segment is already present when its ectoderm begins to develo By the time the ectoderm of the segment is formed, a pair of coelomic spac has appeared in the mesodermal bands at the same level (*cme*, fig. 5, A). Th relationship between ectoderm and mesoderm obtains for all the embryon segments and segmentation can therefore be recognized in the mesoder much sooner than in the ectoderm (fig. 2). The 4d teloblasts continue to ac cells to the mesodermal bands and at the same time further paired coelom cavities (*c 1*, *c 2*, *c 3*, &c.) appear in the bands, evenly from before backwar (figs. 5, A–C; 6, C, D; 7, A–D; 8, A). As the cavities enlarge their walls form th somatic, splanchnic, and anterior and posterior septal mesoderm of successiv segments. The cells of the septa, at first irregularly arranged, later form tw distinct epithelia, and only at this stage (fig. 6, D) are definite segment somites recognizable. The cavities also extend dorsally and ventrally as th mesoderm grows towards the mid-line above and below the endoderm (fig. 1 A, B, p. 130) and double epithelial dorsal and ventral mesenteries are forme (*dms*, *vms*, fig. 17, D). When the somites of the twelfth trunk segment hav been laid down on the eighth day, mesoderm formation ceases and does no begin again until after metamorphosis (figs. 2; 7, C; 8, B; 9, A).

The later history of the 4d teloblasts is complicated by the development the endoderm. For the first 5 days of trunk formation the endodermal tub lengthens by cell-division at a rate consonant with the increase in length of th trunk (*en*, figs. 5, A–C; 6, C, D; 7, A–D; 9, C, D; 10). On the seventh day development, however, division becomes much more pronounced in th posterior endoderm cells and the endodermal tube grows back between th ends of the mesodermal bands to make contact with the pygidial ectoder

FIG. 6. A, 32-day pre-adult, lateral view. B, 40-day pre-adult, lateral view. C, early 6-da embryo, diagrammatic frontal section. D, late 4-day embryo, diagrammatic frontal section *ac*, anal cirrus; *akr*, akrotroch rudiment; *ann*, post-prostomial annulus; *c 5*, *c 7*, coelom cavities of chaetigers 5, 7; ca_1, ca_2, combined coelomic cavities ca_1 and ca_2; ca_3, third pair lateral coelomic cavities of prototrochal region; *cga*, anterior rudiment of cerebral ganglio *ch 1*, *ch 2*, &c., chaetiger 1, 2, &c.; *chs 1*, *chs 2*, *chs 3*, chaetal sacs of chaetigers 1, 2, 3; *db 1 db 18*, dorsal branchia of chaetiger 10, 18; *dbc*, branchial cilia; *dc*, dorsal cirrus; *et*, ectotel blast; *g 1*, *g 2*, gastrotrochs of chaetigers 1, 2; *is 3*, *is 4*, intersegmental septa behind chaetige 3, 4; *ll*, lower lip; *lu*, upper lip; *m*, mouth; *me*, metatroch; *mes*, metatrochal segment; *m* mouth region; *mt*, mesodermal teloblast; *ne 2*, *ne 9*, neuropodium of chaetiger 2, 9; *no no 9*, notopodium of chaetiger 2, 9; *pra*, proboscis apparatus; *pro*, prostomium; *py*, pygidium *t*, telotroch.

pro
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no2
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dc
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ch9
no9
ch10
ac
py
ch16
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200μ
pro
ann
Lu
ll
ch1
mes
ch4
ch10
db18
py
ch19
B
me
chs1
chs2
g1
g2
chs3
is3
is4
t
et
c7
mt
C
100μ
cga
ca3
ca2 a2
akr
pra
ch1
17B
et
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c5
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D

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cga
ca1a2
ak
cgp
ca3
cme
chs I
17C
9B
chs 4
chs 5
A
100μ
et
c9
mt
c8
se
c9
et
pye
mt
enp
et
tc
B
30μ
enp
et
t
bs
so 9
so 10
so 11
C
100μ
12B
cn
cga
div I
cgp
prm st
p
prc
ca0me
ist
me
chs I
en
chs 8
chs 9
et
c11
rm

p, figs. 7, C, D; 12, A). The mesodermal bands are separated by the endo-
rm only along the central axis of the embryo, and remain in contact above
d below it. Consequently the mesodermal mesenteries in the more posterior
gments are formed in position and not as a result of dorsal and ventral
owth (fig. 7, B). By the time the endoderm meets the pygidial ectoderm (*pye*)
e last pair of embryonic trunk somites has been laid down and the meso-
rmal teloblasts are indistinguishable in the cylinder of residual mesoderm
ls which surrounds the posterior end of the gut (*rm*, figs. 7, C, D; 9, A). The
idual mesoderm, like the ectoteloblast ring, persists unchanged throughout
tamorphosis (*rm*, figs. 8, A; 9, A; 14, A). It is formed entirely from descend-
ts of 4d.

Thus in the formation of the embryonic trunk, segmentation of the meso-
rm precedes that of the ectoderm. Each of the 12 segments successively laid
wn contains a pair of hollow somites descended from 4d. The somites sur-
und a common endodermal tube and are covered externally by segmental
toderm, thicker ventrally than dorsally, descended from 2d. Residual 4d
esoderm and 2d ectoderm persist in the prepygidial growth zone.

rther development and metamorphosis

While the embryonic trunk segments are being laid down development
ntinues in the remainder of the embryo, and at the same time the newly
med segments begin to differentiate. By the fifteenth day after spawning,
tamorphosis is complete and every part of the embryo has assumed its
nctional pre-adult condition. The developmental history of each part will
w be described in detail.

The prostomium. The ectoderm of the prostomium arises from part of the
ical plate of the trochophore. From an early stage radial division occurs in
the apical plate cells (*ap*) except those lying immediately anterior to the
ototroch (*p*), which remain a single-layered epithelium (fig. 5, C), and the
ical plate increases in depth and becomes pointed anteriorly (*ap*, figs. 5, B;
C; 10). At the same time the two eyespots are formed as lateral cups of
gment in cells near the prototroch. Their exact mode of origin is obscure.
At the level of the anterior end of the mesoderm a ring of cells with large

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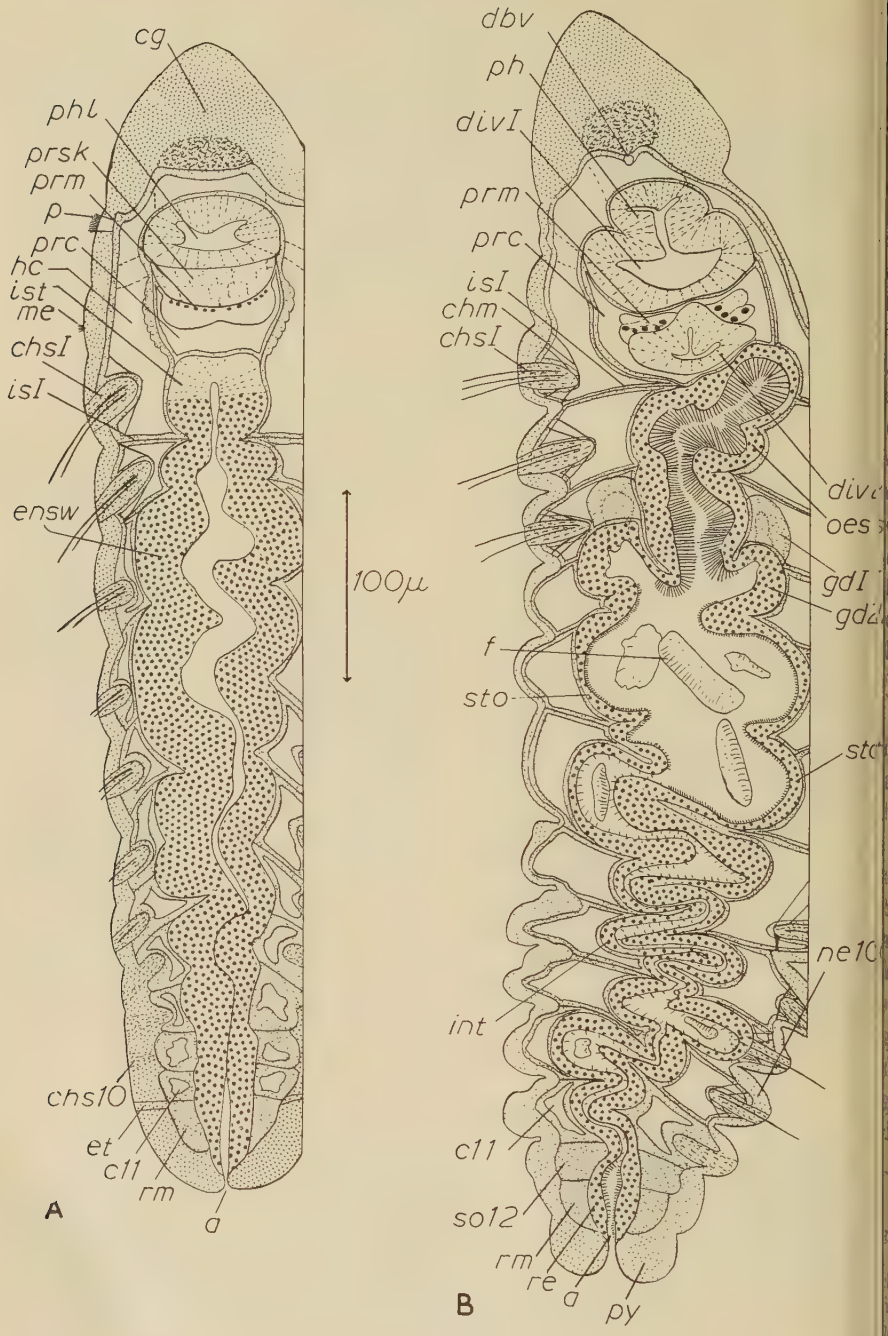
IG. 7. A, diagrammatic frontal section of embryo of fig. 1, H. B, frontal section of posterior
of embryo of fig. 1, H. C, frontal section of posterior end of embryo of fig. 3, A. D, dia-
mmatic frontal section of embryo of fig. 3, A.

, anus; *ak*, akrotroch; *bs*, blood sinus; *c 7*, *c 8*, &c., coelomic cavities of chaetigers 7, 8, &c.;
me, combined coelomic cavities of prototrochal region and metatrochal segment; ca_1a_2,
bined coelomic cavities ca_1 and ca_2; ca_3, third pair of lateral coelomic cavities of proto-
hal region; *cga*, anterior rudiment of cerebral ganglion; *cgp*, posterior rudiment of cerebral
glion; *chs 1*, *chs 4*, &c., chaetal sacs of chaetigers 1, 4, &c.; *cme*, coelomic cavity of meta-
hal segment; *cn*, neuropile of cerebral ganglion; *div 1*, first pharyngeal diverticulum; *en*,
oderm; *enp*, posterior endoderm; *et*, ectoteloblast; *ist*, inner end of stomodaeum; *me*,
atroch; *mt*, mesodermal teloblast; *p*, prototroch; *prc*, proboscis cavity; *prm*, proboscis
actor muscle; *pye*, pygidial ectoderm; *rm*, residual mesoderm; *se*, segmental ectoderm;
, *so 10*, *so 11*, somites of chaetigers 9, 10, 11; *st*, stomodaeum; *t*, telotroch; *tc*, telotroch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al nuclei, yolky inner cytoplasm, and granular outer cytoplasm differentiates the apical plate (*akr*, figs. 6, D; 9, C; *ak*, fig. 10). These are the cells of the otroch. They attain their maximum development on the seventh day . 11, B) but begin to regress on the eighth (fig. 13, B) and are completely orbed by the tenth. The akrotroch cells separate the anterior, radially iding apical plate cells from the posterior single layer of apical plate oderm (figs. 6, C, D; 8, A; 9, C, D; 12, A; 14, A).

In front of the akrotroch radial division of the cells continues dorsally and erally, but ventrally it ceases early. By the time the akrotrochal cilia are sent (*ak*, fig. 1, D, E) a definitive epidermal layer has been formed on the ntral surface of the apical plate anterior to them. Internally a prostomial nd (*gl*) is differentiated by transformation of some of the apical plate cells o unicellular gland cells (figs. 9, D; 11, B). The gland persists throughout tamorphosis (*gl*, figs. 12, A, B; 14, A; 15, B, C). Behind it a transverse row ciliated cells develops in the ventral ectoderm (*cil*, figs. 9, D; 12) but soon appears (compare figs. 4–7). The anterior apical plate ectoderm also in- des a group of cells running from the outer surface through the mass of ively dividing cells to the mid-point of the inner surface. Initially yolk- d and undifferentiated, the cells elongate as the apical plate increases in th and develop into prostomial muscles (*prom*, figs. 9, D; 10; 12; 14, A). r a short time they function in retraction of the tip of the apical plate 94). By the end of metamorphosis they have almost disappeared.

By the seventh day the anterior part of the apical plate consists of a large mber of small cells which form the anterior rudiments of the cerebral glion (*cga*). The neuropile of the ganglion is now visible on its inner sur- e, with the prostomial muscles running through it (*cn*, figs. 8, A; 9, D; 11). mmediately posterior to the akrotroch (*ak*) the anterior ends of the develop- dorsal longitudinal muscles (p. 137) grow through the ectoderm and come inserted on the cuticle. At the same time the posterior part of the cal plate, between the akrotroch and the prototroch, begins to increase in gth (compare fig. 6, A, B and p. 94) mainly anterior to the level of the eyes (*e*), lich remain near the prototroch. The ectoderm of the growing region under- s radial divisions dorsally and laterally to form the posterior rudiment of cerebral ganglion (*cgp*), in which the eyes become embedded, and as the glion rudiment grows inwards it also surrounds the anterior ends of the rsal longitudinal muscles (*cgp*, fig. 12, B). Adjacent to the prototroch (*p*)

IG. 8. A, diagrammatic frontal section of embryo of fig. 3, C. B, 16-day embryo, dia- mmatic frontal section.

, anus; *c 11*, coelomic cavity of chaetiger 11; *cg*, cerebral ganglion; *chm*, chaetal sac muscle; *r, chs 10*, chaetal sacs of chaetigers 1, 10; *dbv*, dorsal longitudinal blood vessel; *div 1*, *div 2*, and second pharyngeal diverticula; *ensw*, endodermal swellings; *et*, ectoteloblast; *f*, food; , *gd 2*, first and second gut diverticula; *hc*, head coelom; *int*, intestine; *is 1*, intersegmental um behind chaetiger 1; *ist*, inner end of stomodaeum; *me*, metatroch; *ne 10*, neuropodium haetiger 10; *oes*, oesophagus; *p*, prototroch; *ph*, pharynx; *phl*, pharyngeal lumen; *prc*, boscis cavity; *prm*, proboscis retractor muscle; *prsk*, proboscis skeleton; *py*, pygidium; ectum; *rm*, residual mesoderm; *so 12*, somite of chaetiger 12; *sto*, stomach.

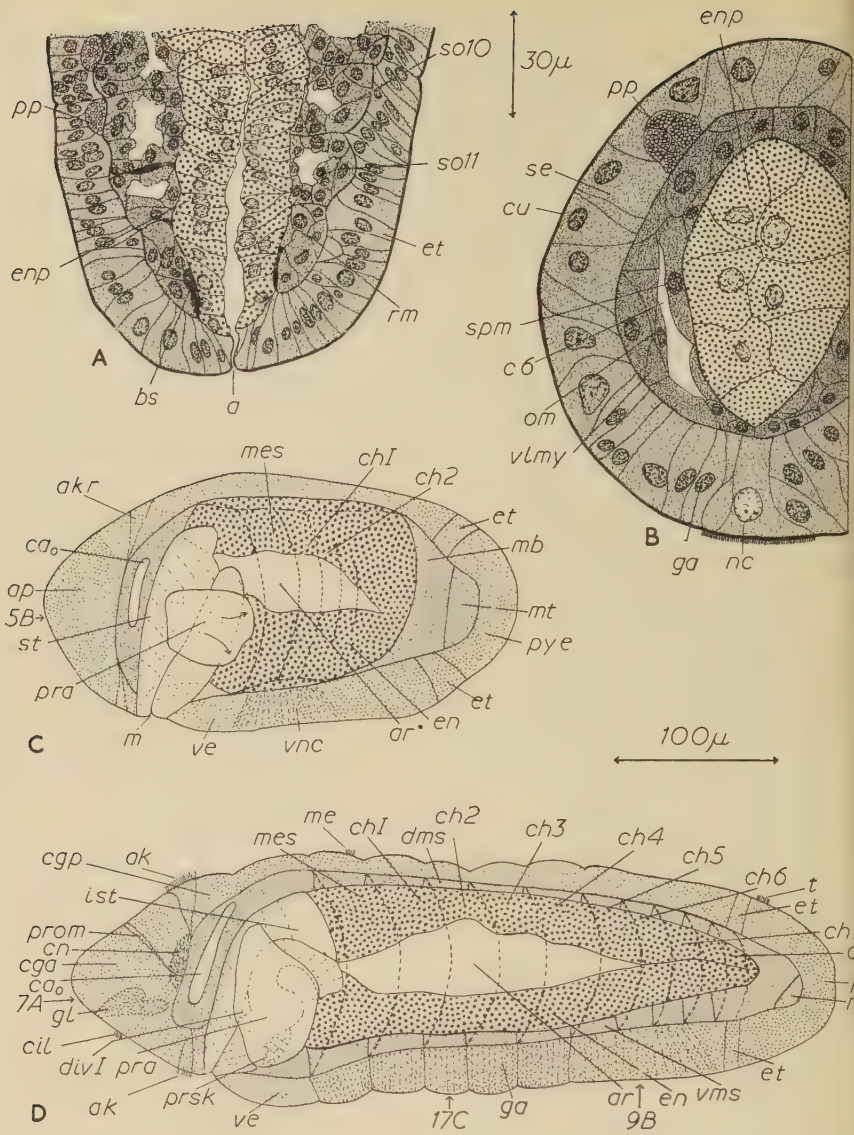


FIG. 9. A, frontal section of posterior end of embryo of fig. 3, C. B, transverse section of chaetiger 6 of embryo of fig. 1, H, at level indicated. C, partial reconstruction, in sagittal plane of embryo of fig. 1, C. D, partial reconstruction, in sagittal plane, of embryo of fig. 1, H. *a*, anus; *ak*, akrotroch; *akr*, akrotroch rudiment; *ap*, apical plate; *ar*, archenteron; *bs*, blood sinus; *c 6*, coelomic cavity of chaetiger 6; ca_0, anterior median coelomic cavity; *cga*, anterior rudiment of cerebral ganglion; *cgp*, posterior rudiment of cerebral ganglion; *ch 1*, *ch 2*, &c., chaetiger 1, 2, &c.; *cil*, cilia; *cn*, cerebral neuropile; *cu*, cuticle; *div 1*, first pharyngeal diverticulum; *dms*, dorsal mesentery; *en*, endoderm; *enp*, posterior endoderm; *et*, ectoteloblast; *gl*, ventral ganglion; *gl*, prostomial gland; *ist*, inner end of stomodaeum; *m*, mouth; *mb*, mesodermal band; *me*, metatroch; *mes*, metatrochal segment; *mt*, mesodermal teloblast; *n*, neurotroch cell; *om*, oblique muscle; *pp*, prototrochal process; *pra*, proboscis apparatus; *prom*, prostomial muscle; *prsk*, proboscis skeleton; *pye*, pygidial ectoderm; *rm*, residual mesoderm; *se*, segmental ectoderm; *so 10*, *so 11*, somites of chaetigers 10, 11; *spm*, splanchnic mesoderm; *st*, stomodaeum; *t*, telotroch; *ve*, ventral ectoderm; *vlmy*, myoblasts of ventral longitudinal muscle; *vms*, ventral mesentery; *vnc*, ventral nerve-cord.

wth is not accompanied by radial divisions, so that a band of apical plate oderm separates the prototroch from the cerebral ganglion. Ventrally a ple increase in area occurs of the ectoderm between the akrotroch and mouth.

The prostomial annulus develops on the seventh day at the posterior border he cerebral ganglion (*ann*, fig. 1, H, I). The apical plate ectoderm behind s level forms part of the ectoderm of the mouth region (figs. 7, D; 13, B). e akrotroch (*ak*) is first resorbed ventrally on the eighth day and at the he time the prostomial annulus extends downwards between its ends in h a way that part of the ventral ectoderm which originally lay in front of akrotroch, as well as the ectoderm behind it, becomes posterior to the ulus. All of this ectoderm is incorporated into the upper lip of the mouth fig. 12, B). As the prostomium becomes externally delineated, it decreases size, owing to differentiation of the ganglion cells, which become smaller more compactly arranged, and to resorption of the akrotroch (*cga*, *cgp*, . 11; 12, B; 13, B; and *cg*, fig. 15, C). With the final disappearance of the otroch the cerebral ganglion becomes a single morphological unit. The ropile of the ganglion is produced into two lateral horns which grow back ween the ectoderm and mesoderm of the mouth region (*cph*, fig. 14, B, C) circumpharyngeal commissures and join up with the neuropile of the tral nerve cord just before hatching (p. 132). Between hatching and the of metamorphosis the cerebral ganglion again increases in size and separ- s from a superficial layer of epidermal cells. Apically the superficial dermis becomes several cells thick, forming the pointed anterior end of the stomium characteristic of the adult.

The apical plate is lined internally by mesoderm from an early stage in elopment (figs. 5, B, C; 9, C; 10). On the seventh day the mesoderm begins grow forward ventrally between the neuropile (*cn*) and akrotroch (*ak*), arating the ganglion rudiment (*cga*) from the ventral ectoderm (fig. 9, D; B). By the end of metamorphosis it has extended as far as the anterior gin of the upper lip (*pm*, fig. 15, B, C) but in front of this the cerebral glion and ventral ectoderm remain in contact. No clear distinction can be wn between the mesoderm of the prostomium and that of the mouth ion and for this reason their development is discussed together on pp. 123–9.

The ectoderm of the mouth region. The ectoderm of the mouth region is nposite in origin. Anterior to the prototroch it arises from apical plate cells 104). In the mid-ventral gap in the prototroch in front of the mouth its s are derived from 2b, while in the mid-dorsal gap and behind the proto- h the ectoderm of the mouth region is formed by those 2d cells, other than ectoteloblasts, which cover the embryo during blastoporal closure (p. 104; *ve*, *le*, fig. 4, A, B).

n the early stages of development the prototroch cells (*p*) occupy a large t of the surface of the future mouth region (fig. 5, C). Once the definitive nber of prototroch cells has been attained (p. 104), their nuclei become e and oval with one or two nucleoli, the yolk globules disappear from the

outer parts of the cells, and vacuoles form in the cytoplasm. The cilia fir project from the prototroch cells in the trochophore (fig. 4, C) and the increase in length to a maximum of 40μ (fig. 11, A). On the seventh day th cells begin to degenerate. Their cilia show no differential growth or organiz tion into rows.

The four posterior primary prototroch cells on each side undergo a furth change. On the fourth day the inner end of each one begins to grow as process filled with yolk globules. The processes of the two dorsal cells gro directly back between the ectoderm and mesoderm dorso-laterally on each sid of the embryo. Those of the more ventral cells grow first upwards to meet th dorsal processes and then back with them (figs. 9, B; 13, A; 17, C) and sho a number of irregular swellings and lateral branches, the details of whic vary in different embryos. With the recession of the prototroch the processe break up into isolated yolk masses and finally, just after hatching, vanis without trace (figs. 14, C, D; 19, B). Their function remains obscure. Delsma (1916) mentions the early stages of their development, stating that each h an anterior nucleus; but this is not so. As far as is known at present, no suc outgrowths of the prototroch cells occur in other polychaetes.

Degeneration proceeds more quickly in the posterior prototroch cells tha in the anterior. The cells and their nuclei become smaller and abnormal i appearance (*p*, figs. 7, A, D; 8, A; 13, B) and by the twelfth day are entirel resorbed. At the same time the mouth region decreases in size (*mr*, figs. H, I; 3, A–C). Laterally the anterior apical plate ectoderm meets the posteri 2d ectoderm to form a continuous epidermal layer (fig. 8). Dorsally most the anterior ectoderm is incorporated into the prostomium and little, if an combines with the posterior ectoderm to form the dorsal epidermis of th mouth region. Ventrally the ectoderm behind the mouth folds forward t form the lower lip (*ll*, figs. 9, D; 12; 15, C). The anterior end of the neurotroc is carried forward with it but is later replaced by ectoderm as the neurotroc cells regress. The ventral ectoderm anterior to the mouth forms the upp lip, which meets the prostomium anteriorly (p. 115; *lu*, figs. 9, C, D; 10; 11, E 12; 14, A; 15, C). Thus by the end of metamorphosis the mouth region i covered dorsally and laterally by a simple epithelium one cell thick, whil ventrally its ectoderm has formed the lips of the mouth (figs. 14, C; 15, C). A the cells have lost their yolk contents and some have differentiated into uni cellular gland cells.

The stomodaeum and proboscis apparatus. In the trochophore the stomo daeum and proboscis apparatus are not distinct (p. 104), but by the beginnin of the fourth day the median vertical stomodaeal rudiment (*st*) is differentiate from the two lateral rudiments of the proboscis apparatus (*pra*, figs. 5, B, C 9, C). The uniformly yolky nature of the cells in the early stages preclude a precise analysis of the separate origins of the two organs, but the major par of the proboscis apparatus is formed from descendants of the posterior ecto mesoderm cells (*emp*, fig. 4, A, C) while the cells of the ingrowing rudimen *stpr* form the stomodaeum and also contribute to the proboscis apparatus.

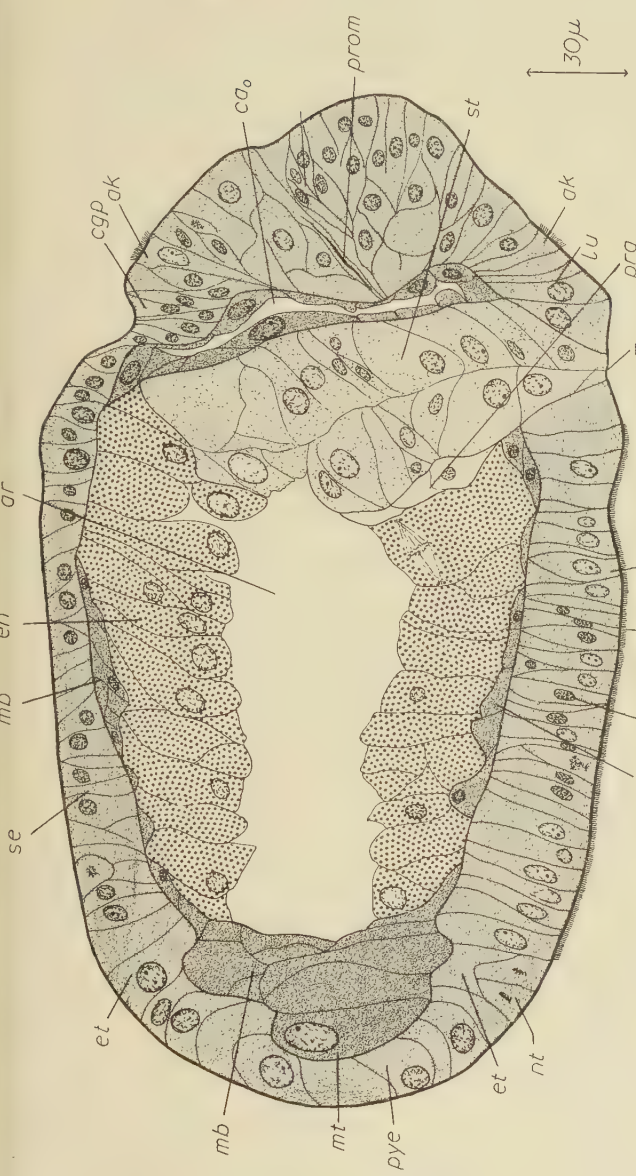


FIG. 10. Sagittal section of embryo of fig. 6, D.

ak, akrotroch; *ar*, archenteron; *ca*, anterior median coelomic cavity; *cgp*, posterior rudiment of cerebral ganglion; *en*, endoderm; *et* ectoteloblast; *ga*, ventral ganglion; *lu*, upper lip; *m*, mouth; *mb*, mesodermal band; *mt*, mesodermal teloblast; *n*, neurotroch; *nc*, neurotroch cell; *nt*, neurotroch teloblast; *pra*, proboscis apparatus; *prom*, prostomial muscle; *pye*, pygidial ectoderm; *se*, segmenta ectoderm; *st*, stomodaeum.

The stomodaeum in *S. armiger* gives rise to the pharynx and the anterio end of the oesophagus. It consists at first of a simple column of yolk-fille cells arranged radially around a central axis and lying wholly within the prot trochal region (*st*, figs. 5, B, C; 9, C). At its inner end (*ist*) is a pair of large cell which remain conspicuous until the end of metamorphosis and mark th junction between the stomodaeum and the endoderm.

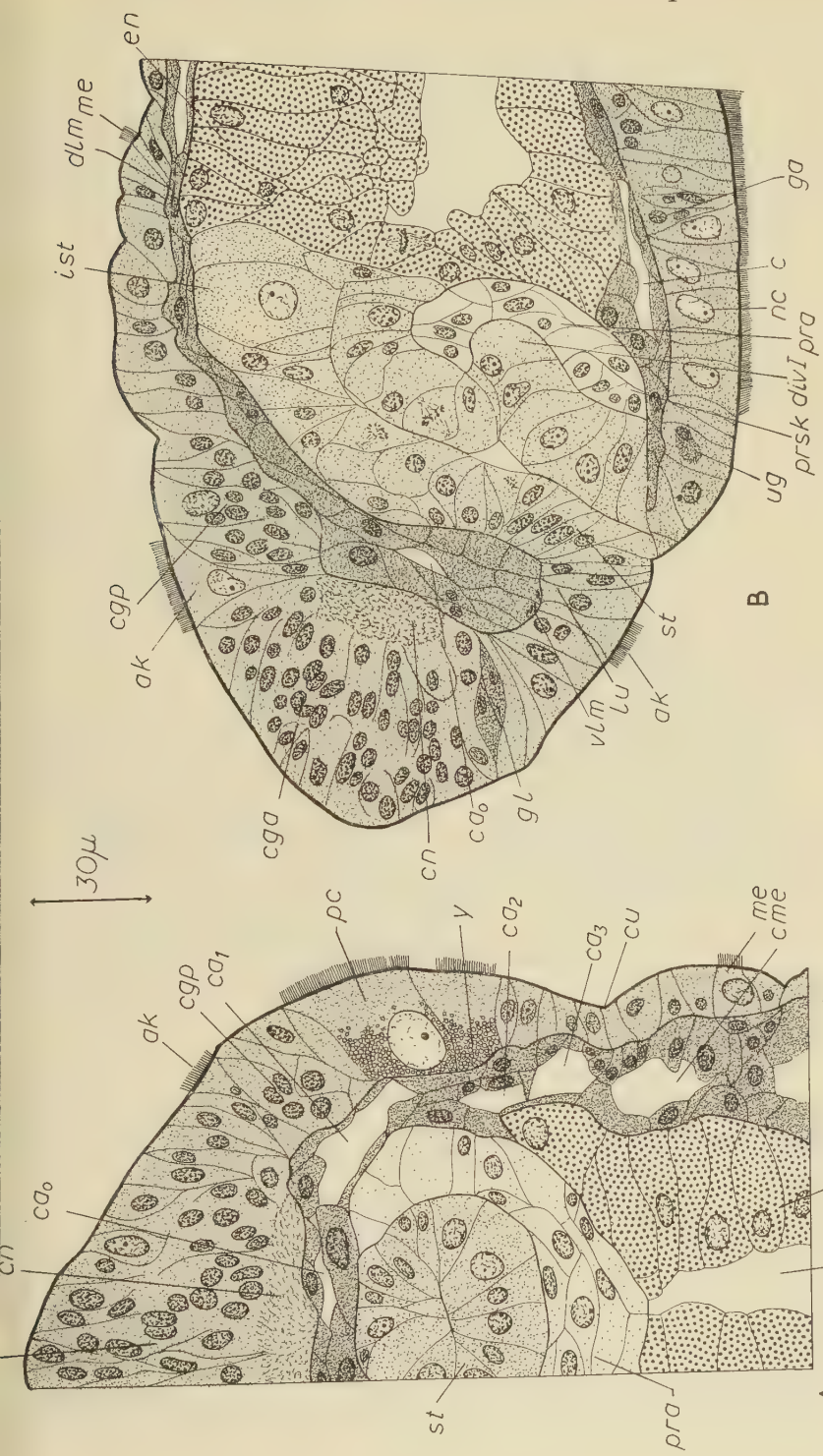
The stomodaeum grows first dorsally, then from the sixth day in a posterio direction (*st*, figs. 9, D; 10; 11, B). Meanwhile changes also occur in the rud ments of the proboscis apparatus. During the fourth day they begin to exten towards the mid-line between the stomodaeum (*st*) and the ventral and latera endoderm (*en*, *pra*, figs. 5, B, C; 9, C) and soon cover the lateral and posterio surfaces of the stomodaeum (*pra*, figs. 5, D; 10; 11, A). When the inner en of the stomodaeum turns posteriorly its ventral wall emerges above the dorsa margin of the rudiment of the proboscis apparatus (*pra*) and grows downward to establish contact with the up-growing ventral and median-growing latera endoderm (fig. 9, D; 11, B). Continued growth of the stomodaeum carries it inner end (*ist*) through the metatrochal segment into chaetiger 1, the anterio end of the endodermal tube passing back with it (fig. 12).

By the seventh day yolk has disappeared from the inner parts of th stomodaeal cells but still remains in the outer parts. The virtual lumen firs opens as a transverse slit at the point where the first pharyngeal diverticulum develops (*div 1*, figs. 9, D; 11, B). By the time continuity has been establishe between the stomodaeum and the endodermal tube, the lumen extends from the now open mouth to the point where the large yolky cells mark the junctio of the two components of the gut (*ist*, fig. 12, B). The stomodaeal lumen i lined by a thin cuticle which stains identically with the external cuticle an is continuous with it over the lips of the mouth.

By the end of metamorphosis the mouth is wide and functional. The dis tinction between the lower lip and the posterior wall of the stomodaeum i retained throughout, but in the upper lip the ectoderm and stomodaeal tissu become indistinguishable as the epithelium of the lip becomes more than on cell thick (*lu*, figs. 12, B; 15, C). The anterior and lateral walls of the proxima stomodaeum also thicken and by the end of metamorphosis three prominen folds of epithelium project into the lumen (fig. 8, B). Elsewhere the epithelium of the stomodaeum remains one cell thick. The yolk gradually disappear from the cells towards the end of metamorphosis.

FIG. 11. A, frontal section of anterior end of embryo of fig. 1, H. B, sagittal section of anterior end of embryo of fig. 1, H.

ak, akrotroch; *c*, coelom; ca_0, anterior median coelomic cavity; ca_1, ca_2, ca_3, paired lateral coelomic cavities of prototrochal region; *cga*, anterior rudiment of cerebral ganglion; *cgp*, posterior rudiment of cerebral ganglion; *cme*, coelomic cavity of metatrochal segment; *cn*, cerebral neuropile; *cu*, cuticle; *div 1*, first pharyngeal diverticulum; *dlm*, dorsal longitudinal muscle; *en*, endoderm; *ga*, ventral ganglion; *gl*, prostomial gland; *ist*, inner end of stomodaeum; *lu*, upper lip; *me*, metatroch; *nc*, neurotroch cell; *pc*, prototroch cell; *pra*, proboscis apparatus; *prsk*, proboscis skeleton; *st*, stomodaeum; *vlm*, ventral longitudinal muscle; *ug*, unicellular gland; *y*, yolk globules.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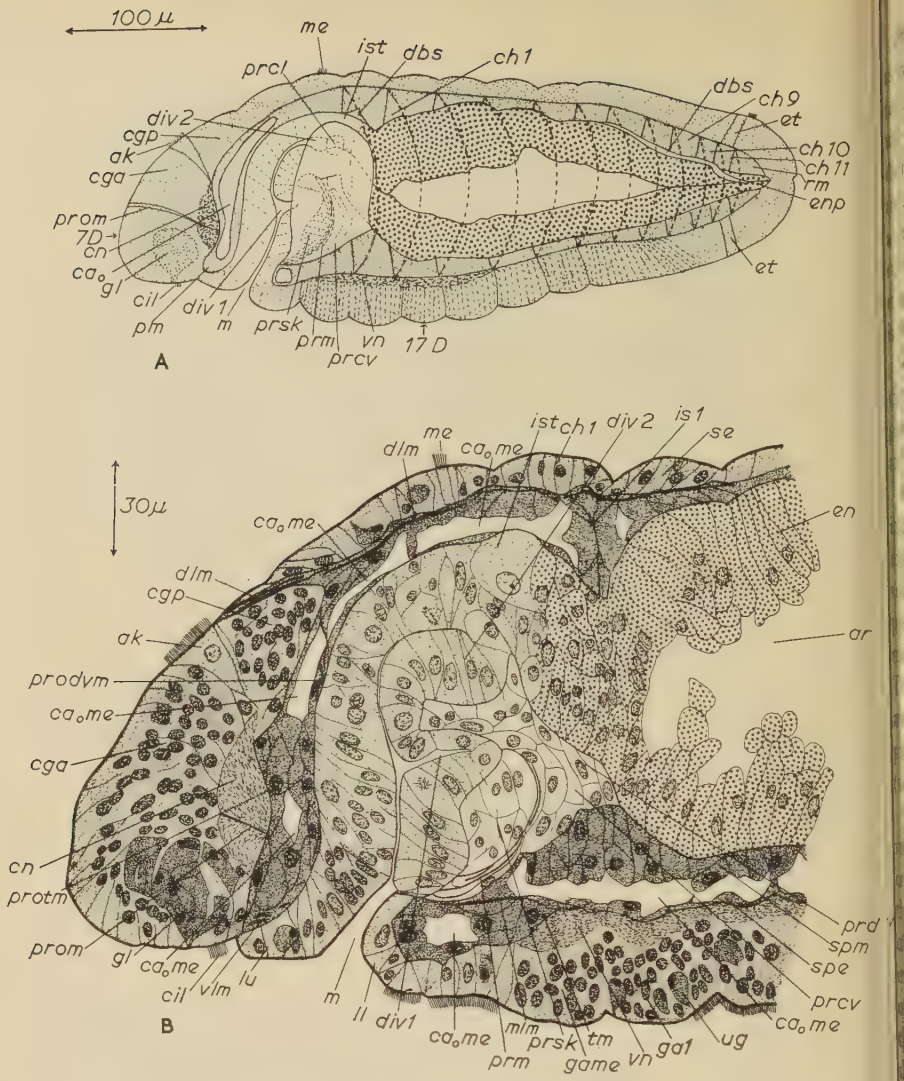

FIG. 12. A, partial reconstruction, in sagittal plane, of embryo of fig. 3, A. B, sagittal section of anterior end of embryo of fig. 3, A.

ak, akrotroch; *ar*, archenteron; ca_0, anterior median coelomic cavity; ca_0me, combined coelomic cavities of prototrochal region and metatrochal segment; *cga*, anterior rudiment of cerebral ganglion; *cgp*, posterior rudiment of cerebral ganglion; *ch 1*, *ch 9*, &c., chaetiger 1, 9, &c.; *cil*, cilia; *cn*, cerebral neuropile; *dbs*, dorsal blood sinus; *div 1*, *div 2*, first and second pharyngeal diverticula; *dlm*, dorsal longitudinal muscle; *en*, endoderm; *enp*, posterior endoderm; *et*, ectoteloblast; *ga 1*, ventral ganglion of chaetiger 1; *game*, ventral ganglion of metatrochal segment; *is 1*, intersegmental septum behind chaetiger 1; *ist*, inner end of stomodaeum; *ll*, lower lip; *lu*, upper lip; *m*, mouth; *me*, metatroch; *mlm*, median longitudinal muscle; *pm*, prostomial mesoderm; *prcl*, lateral cavity of proboscis apparatus; *prcv*, ventral cavity of proboscis apparatus; *prd*, dorsal wall of proboscis cavity; *prm*, proboscis retractor muscle; *prodvm*, dorso-ventral prostomial muscle; *prom*, prostomial muscle; *protm*, transverse prostomial muscle; *prsk*, proboscis skeleton; *rm*, residual mesoderm; *se*, segmental ectoderm; *spe*, somatic peritoneum; *spm*, splanchnic mesoderm; *tm*, transverse muscle; *ug*, unicellular gland; *vlm*, ventral longitudinal muscle; *vn*, neuropile of ventral nerve-cord.

On the eighth day the second pharyngeal diverticulum begins to develop an outgrowth of the ventral wall of the stomodaeum in the metatrochal gment (*div 2*, fig. 12). As it becomes conspicuous the cells of its anterior ll secrete a thick cuticular shield. Posterior to the second pharyngeal verticulum the stomodaeum is not lined by cuticle. The stomodaeal lumen d archenteron become confluent at the hatching stage, and at the same time ing thickening differentiating just behind the second pharyngeal diverti- lum separates the future pharynx from the oesophagus (*oes*, fig. 15, C), part which is formed from stomodaeal tissue. Differentiation of the oesophagus discussed on p. 132.

Soon after hatching short cilia appear on the dorsal and lateral walls of the tal part of the pharynx and on the lateral and posterior walls of the second aryngeal diverticulum, projecting through the cuticle (fig. 15, C). The re- inder of the pharynx is not ciliated.

After fusing in the mid-line the rudiment of the proboscis apparatus reases in size and spreads dorsally over the sides of the stomodaeum, but ferentiation of its various parts does not begin until the seventh day. Then, ntral to the first pharyngeal diverticulum (*div 1*), the cells in contact with the sterior wall of the stomodaeum become arranged in a transverse compact ss (*prsk*, figs. 9, D; 11, B; 12; 13, B), forming the skeleton of the proboscis. e cells lose most of their yolk at an early stage and have clear cytoplasm d darkly staining nuclei. A number of transverse fibres also appear in the boscis skeleton shortly after hatching, but their exact origin and their nature eletal or muscular) remain obscure (*trf*, figs. 14, C; 15, C).

After the first appearance of the proboscis skeleton (*prsk*), the cells im- diately posterior to it begin to elongate dorso-ventrally in two groups, one each side of the median line. Their upper ends become attached either to down-growing second pharyngeal diverticulum (*div 2*) or to the surface the proboscis skeleton, while their lower ends grow round the latter and ert on the posterior wall of the stomodaeum where it meets the ectoderm the lower lip (*prm*, figs. 12; 13, A). In doing so they separate the surface of proboscis skeleton and stomodaeum from the overlying splanchnic meso- m. Muscle-fibres first appear in these cells late on the seventh day, gradu- becoming more conspicuous (*prm*, figs. 12, B; 14, D; 15, C; 19, A), until the end of metamorphosis all yolk globules have disappeared and the cells m two paramedian groups of muscles, the retractor muscles of the pro- scis. These, together with the prostomial muscles, are the only muscles in *armiger* which do not develop from 4d mesoderm.

As the retractor muscles are beginning to differentiate, a pair of lateral rities appears in the upper part of the proboscis apparatus rudiment (*prcl*, s. 12, A; 13, A) and soon after this the layer of cells which overlies the veloping muscles separates from them to leave a third, median cavity below oesophagus (*prcv*, fig. 12). The lateral and median cavities then run ether so that the proboscis skeleton and retractor muscles lie in a space unded on all sides by cells of the proboscis apparatus (*prc*, figs. 8; 14, A, D;

A
dms
ist
dvm
prcl
pp
mec
prmy
game
vn
h
vms
tm
dlm
om
vlm
cm
cme
ug
spm
B
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prodvm
ca1
cga
aom
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cgp
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ca2me
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prm
vms
prmy
me
prcv
30μ

B, C). The dorsal wall of the cavity is apposed to the pharynx and oesophagus (*prd*, figs. 12, B; 15, C). The ventral wall forms with the splanchnic soderm a double epithelial layer separating the proboscis cavity from the lom (*prv*, fig. 19, A).

As the oesophagus differentiates a median diverticulum of the proboscis ity grows back mid-ventrally between it and the splanchnic mesoderm *div*, figs. 14, A; 15, B, C; 19, B). By the end of metamorphosis the diverticum extends to the posterior end of the oesophagus. All yolk has now disppeared from the cells of the proboscis apparatus.

Backward and forward movement of the proboscis is first seen in the 8-day bryo when the contractile fibres in the retractor muscles are still very thin; protrusion and withdrawal of the proboscis does not begin until the day er hatching (fig. 15, C). Protrusion always occurs during circular muscle traction at the anterior end of the embryo, and withdrawal during longitinal muscle contraction. It appears that the circular muscles produce in coelomic fluid a hydrostatic pressure which is transmitted through the d of the proboscis cavity to the proboscis skeleton. The skeleton (*prsk*) is shed out through the mouth, with extension of the lower lip fold (*ll*) and t pharyngeal diverticulum (*div 1*) (fig. 15, B, C). Withdrawal of the proboscis ults from contraction of the longitudinal body-wall muscles, the proboscis ractor muscles, and a number of special muscles (p. 127) inserted on the er lip. The thick anterior wall of the second pharyngeal diverticulum apers to serve as a skeletal support for the proboscis retractor muscles.

The posterior diverticulum of the proboscis cavity possibly functions in nsmitting coelomic pressure from chaetigers 2 and 3, isolated by intermental septa from the head coelom and from each other (fig. 8, B), to the boscis skeleton, thereby increasing its protrusion efficiency.

The mesoderm of the mouth region. As elongation of the trunk begins the erior ends of the mesodermal bands pass between the apical plate (*ap*) and developing stomodaeum (*stpr*) and meet in the mid-line below the anterior omesoderm (*ema*) (fig. 5, A). Then as a result of dorsal and ventral growth

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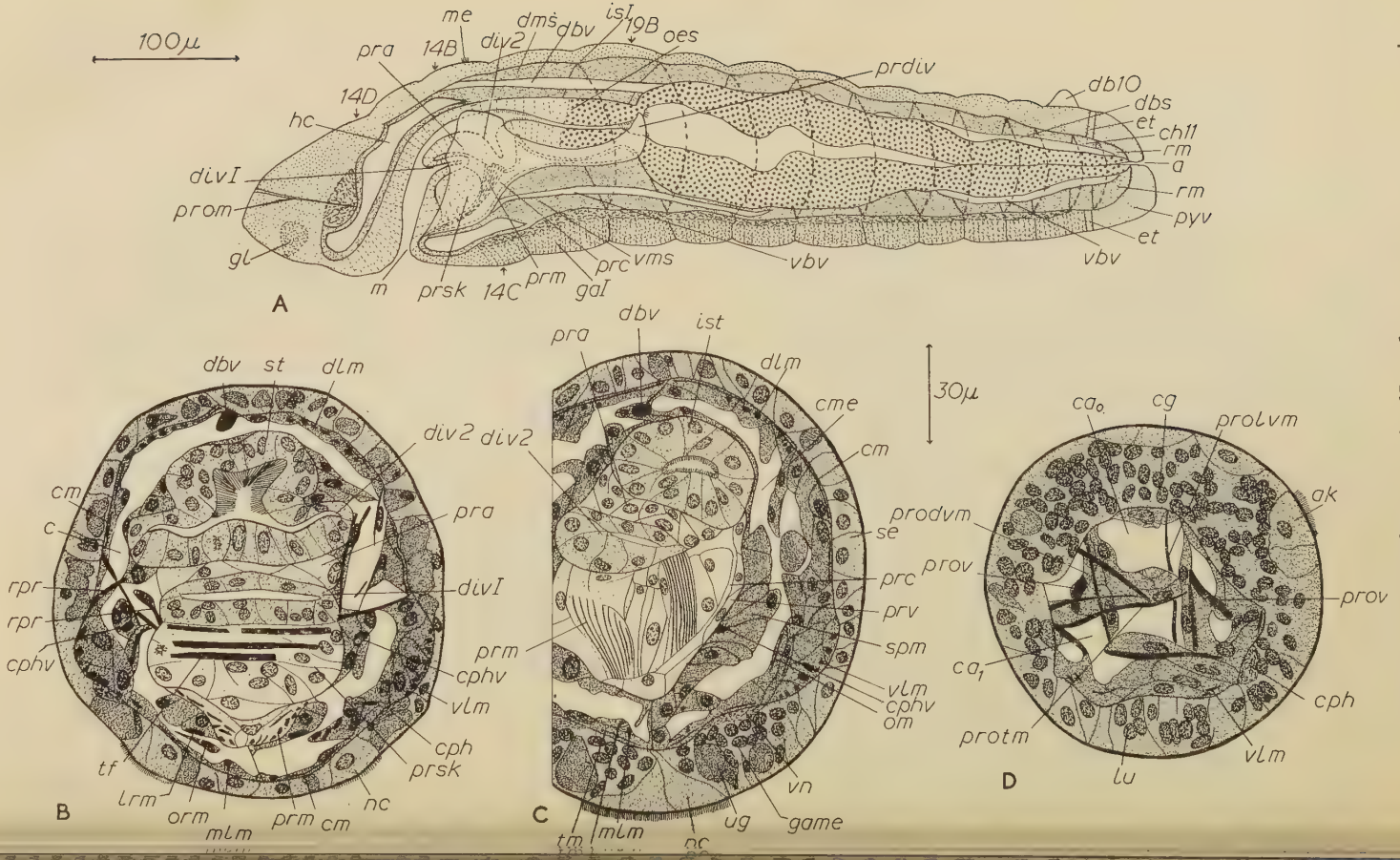
IG. 13. A, late 7-day embryo, transverse section through metatroch. B, 9-day embryo, tal section of anterior end.

, akrotroch; *aom*, anterior oblique muscle; *c 1*, coelomic cavity of chaetiger 1; ca_1, ca_2, two pairs of lateral coelomic cavities of prototrochal region; ca_2me, combined second and d paired coelomic cavities of prototrochal region and cavity of metatrochal segment; *cga*, rior rudiment of cerebral ganglion; *cgp*, posterior rudiment of cerebral ganglion; *ch 1*, tiger 1; *cm*, circular muscle; *cme*, coelomic cavity of metatrochal segment; *cn*, cerebral opile; *dlm*, dorsal longitudinal muscle; *dms*, dorsal mesentery; *dvm*, dorsoventral cle; *game*, ventral ganglion of metatrochal segment; *is 1*, intersegmental septum behind tiger 1; *isme*, intersegmental septum behind metatrochal segment; *ist*, inner end of odaeum; *me*, metatroch; *mec*, metatroch cell; *mes*, metatrochal segment; *mr*, mouth on; *n*, neurotroch; *om*, oblique muscle; *p*, prototroch; *pp*, prototrochal process; *prcl*, al proboscis cavity; *prcv*, ventral proboscis cavity; *prm*, proboscis retractor muscles; , myoblasts of proboscis retractor muscles; *prodvm*, dorso-ventral prostomial muscles; , proboscis skeleton; *spm*, splanchnic mesoderm; *st*, stomodaeum; *tm*, transverse muscle; unicellular gland; *vlm*, ventral longitudinal muscle; *vms*, ventral mesentery; *vn*, neuropile entral nerve-cord.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mesoderm of each side fuses in the mid-line above the stomodaeum and doderm and below and behind the stomodaeum and proboscis apparatus gs. 9, D; 11, B).

Formation of the paired coelomic cavities of the trunk segments has already en discussed (p. 108). Those of the metatrochal segment are not, however, most anterior coelomic cavities of the embryo. In the prototrochal region ee pairs of cavities, ca_3, ca_2, ca_1, develop evenly from behind forwards at same time as those of the anterior trunk segments. A median anterior ity ca_0 then appears in the mesoderm in front of the stomodaeum, its lls being formed partly from mesodermal band cells and partly from anior ectomesoderm (*ema*, fig. 5, A, B). As the cavities increase in size, anchnic, somatic, and septal mesoderm are differentiated.

The septa of the prototrochal region are not permanent structures. Those ween ca_1 and ca_2 on each side break down on the fourth day (fig. 6, D), and se between ca_0 and ca_1, ca_2 and ca_3, and ca_3 and the coelomic cavities of the tatrochal segment on the eighth day (figs. 6, C; 7, A, D; 11, A). In each case ne of the cells of the septa persist as myoblasts. The septa between ca_0 and give rise to a number of dorso-ventral muscles connecting the dorsal body-ll of the mouth region to the anterior face of the stomodaeum (*prodvm*, s. 12, B; 13, B; 14, B; 15, C). The more lateral of these muscles merge at their per ends into the dorsal longitudinal muscles. The anterior ends of the erior oblique muscles (*aom*, fig. 10, B) also arise in the same septa and in septa between ca_1 and ca_2, which otherwise disappear completely. In the ta between ca_2 and ca_3 are formed three pairs of muscles connecting postero-lateral wall of the stomodaeum with the lateral body-wall of the uth region. The septa between ca_3 and the metatrochal segment give rise two groups of muscles running from the body-wall to the first pharyngeal erticulum and the proboscis skeleton (*rpr*, fig. 14, C). Their orientation

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IG. 14. A, 11-day embryo, partial reconstruction in sagittal plane. B, transverse section of uth region of embryo of fig. 14, A. C, transverse section of metatrochal segment of embryo ig. 14, A. D, transverse section of prostomium of embryo of fig. 14, A.

, anus; *ak*, akrotroch; *c*, coelom; ca_0, anterior median coelom; ca_1, first pair of lateral lomic cavities of prototrochal region; *cg*, cerebral ganglion; *ch 11*, chaetiger 11; *cm*, ular muscle; *cme*, coelom of metatrochal segment; *cph*, circumpharyngeal nerve commise; *cphv*, circumpharyngeal blood-vessel; *db 10*, dorsal branchia of chaetiger 10; *dbs*, dorsal od sinus; *dbv*, dorsal blood-vessel; *div 1*, *div 2*, first and second pharyngeal diverticula; , dorsal longitudinal muscle; *dms*, dorsal mesentery; *et*, ectoteloblast; *ga 1*, ventral glion of chaetiger 1; *game*, ventral ganglion of metatrochal segment; *gl*, prostomial gland; , intersegmental septum behind chaetiger 1; *ist*, inner end of stomodaeum; *lrm*, longitudiretractors of lower lip; *lu*, upper lip; *m*, mouth; *me*, metatroch; *mlm*, median longitudinal scle; *nc*, neurotroch cell; *oes*, oesophagus; *om*, oblique muscle; *orm*, oblique retractors of er lip; *pra*, proboscis apparatus; *prc*, proboscis cavity; *prm*, proboscis retractor muscle; *dvm*, dorsoventral prostomial muscle; *protm*, transverse prostomial muscle; *prov*, pronial blood vessel; *prsk*, proboscis skeleton; *prv*, ventral wall of proboscis cavity; *pyv*, tral part of pygidium; *rm*, residual mesoderm; *rpr*, lateral retractors of proboscis; *se*, mental ectoderm; *spm*, splanchnic mesoderm; *st*, stomodaeum; *ug*, unicellular gland; *vbv*, tral longitudinal blood-vessel; *vlm*, ventral longitudinal muscle; *vms*, ventral mesentery; neuropile of ventral nerve-cord.

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such that they are stretched when proboscis eversion occurs and assist in boscis withdrawal.

When the coelomic cavities of the prototrochal region come together in the rsal mid-line, the mesentery separating them disappears so that a continu- s coelomic cavity crossed by muscle strands surrounds the stomodaeum teriorly, dorsally, and laterally and is confluent with the coelom of the tatrochal segment (*ca*$_0$*me*, figs. 7, D; 12; 14, A; 15, C). The septum between metatrochal segment and chaetiger 1 also begins to break down at the ne time, but does not disappear completely until the hatching stage 133). It cannot be seen in fig. 12, B, for instance, but part of it remains fig. 13, B, *isme*. The coelom around the stomodaeum gradually extends ow and behind the stomodaeum and proboscis apparatus into the lower . The anterior ends of a number of muscles which differentiate in the tially solid mesoderm of the lower lip (*ll*) and are attached to the inner rgin of the lip below the ends of the proboscis retractor muscles (*prm*), are in position as the coelom spreads round them. They are the median longi- dinal muscles (*mlm*), the longitudinal retractor muscles (*lrm*), and the ique retractor muscles (*orm*) of fig. 16 (see also *mlm*, fig. 12, B; *mlm*, *lrm*, *n*, figs. 14, C; 15, C). Their function is to refold the lower lip during boscis withdrawal. The more posterior parts of the muscles are formed in somatic mesoderm of the embryonic trunk segments (p. 137).

At the time when the prototrochal region is externally divided into pro- mium and mouth region (fig. 3, A, B), the splanchnic mesoderm of the ion forms a thin epithelium covering the developing pharynx and proboscis paratus (fig. 12, B). The somatic mesoderm lining the mouth region, in dition to forming peritoneum, gives rise to a circular muscle-layer (*cm*) mediately below the ectoderm and to the anterior ends of the dorsal and ntral longitudinal muscles (p. 137; *dlm*, *vlm*, fig. 14, C). The former extend o the prostomium (*dlm*, fig. 15, C; p. 113). The latter run lateral to the uth and insert on the upper lip (*vlm*, figs. 12, B; 13, B; 15, C). The behaviour the embryos shows that the body-wall musculature of the mouth region is

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IG. 15. A, diagram to illustrate anterior connexions between dorsal and ventral longitudinal od-vessels of late embryo. B, partial reconstruction, in sagittal plane, of embryo of fig. 3, D. agittal section of anterior end of embryo of fig. 14, A.

g, cerebral ganglion; *ch 1*, chaetiger 1; *cn*, cerebral neuropile; *cphv*, circumpharyngeal od-vessel; *cpro*, prostomial coelom; *cv 1*, *cv 2*, commissural blood-vessels in chaetigers 1, 2; dorsal blood sinus; *dbv*, dorsal blood-vessel; *div 1*, *div 2*, first and second pharyngeal rticula; *dlm*, dorsal longitudinal muscle; *et*, ectoteloblast; *ga 1*, *ga 2*, &c., ventral ganglion haetiger 1, 2, &c.; *gas*, sub-oesophageal ganglion; *gd 1*, first gut diverticulum; *gl*, pro- mial gland; *is 1*, *is 2*, &c., intersegmental septum behind chaetiger 1, 2, &c.; *ist*, inner end tomodaeum; *ll*, lower lip; *llat*, lateral lip; *lrm*, longitudinal retractors of proboscis; *lu*, er lip; *m*, mouth; *me*, metatroch; *mes*, metatrochal segment; *mlm*, median longitudinal scle; *mr*, mouth region; *om*, oblique muscle; *ph*, pharynx; *pm*, prostomial mesoderm; *pras*, od sinus behind proboscis apparatus; *prc*, proboscis cavity; *prm*, proboscis retractor scle; *pro*, prostomium; *prodvm*, dorso-ventral prostomial muscle; *protm*, transverse stomial muscle; *prov*, prostomial blood-vessel; *prsk*, proboscis skeleton; *spm*, splanchnic toderm; *ug*, unicellular gland; *vbv*, ventral longitudinal blood-vessel; *vlm*, ventral longi- inal muscle.

actively contractile before that of the trunk segments (p. 94) but it is ver difficult to confirm from sections that it is the first to develop.

The somatic mesoderm covering the inner surface of the cerebral ganglio forms the mesoderm of the prostomium. It originates from the somatopleur of the anterior median coelomic cavity ca_0 and part of the somatopleure of th

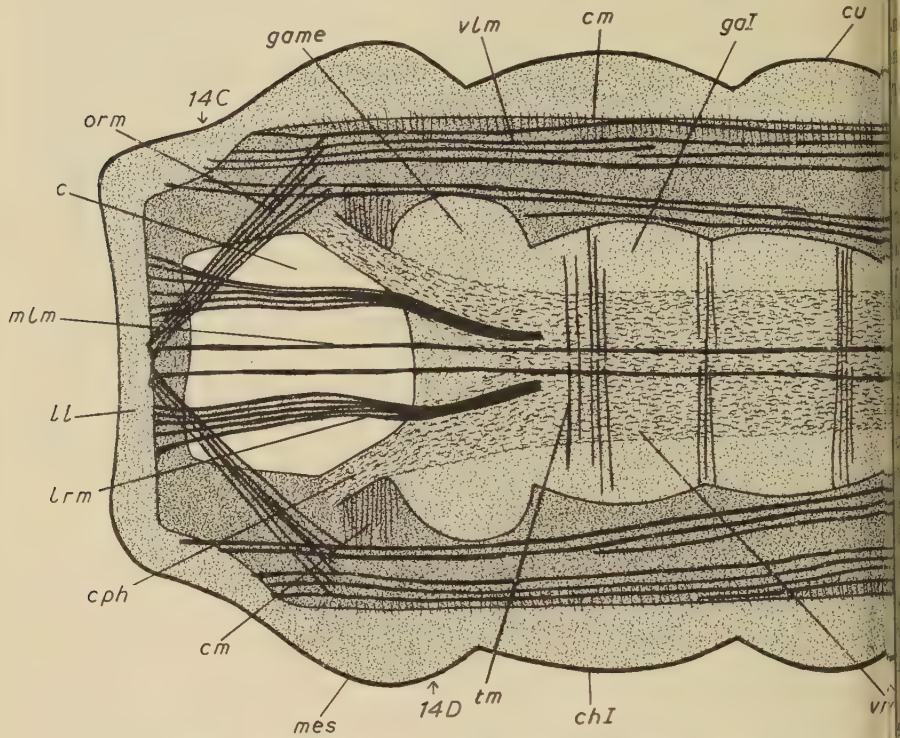


FIG. 16. Diagrammatic frontal section of 11-day embryo to illustrate retractor musculatu of lower lip (not to scale).

c, coelom; *cm*, circular muscle; *cph*, circumpharyngeal nerve commissures; *cu*, cuticle; *ga* ventral ganglion of chaetiger 1; *game*, ventral ganglion of metatrochal segment; *ll*, lower lip *lrm*, longitudinal retractors of lower lip; *mlm*, median longitudinal muscles; *mes*, metatroch segment; *orm*, oblique retractors of lower lip; *tm*, transverse muscle; *vlm*, ventral longitudin muscle; *vn*, neuropile of ventral nerve-cord.

first pair of lateral cavities ca_1 (fig. 7, D). The external annulus separating th prostomium from the mouth region has no equivalent in the mesoderm. O the seventh day muscles begin to differentiate in the prostomial mesoderm some running transversely across the inner surface of the cerebral ganglio (*protm*, figs. 14, B; 15, C), some dorso-ventrally from the inner surface of th neuropile to the developing upper lip (*prodvm*, figs. 13, B; 14, B). As th mesoderm extends forward beneath the cerebral ganglion (p. 115), the dorso ventral muscles are left in position, traversing the coelom.

All the musculature of the prostomium and mouth region is well differen tiated by the time of hatching and undergoes little further change before th

of metamorphosis. Soon after hatching a number of blood-vessels are eloped in the mesoderm of the prostomium and mouth region. They are ussed on p. 135 together with the vascular system of the trunk.

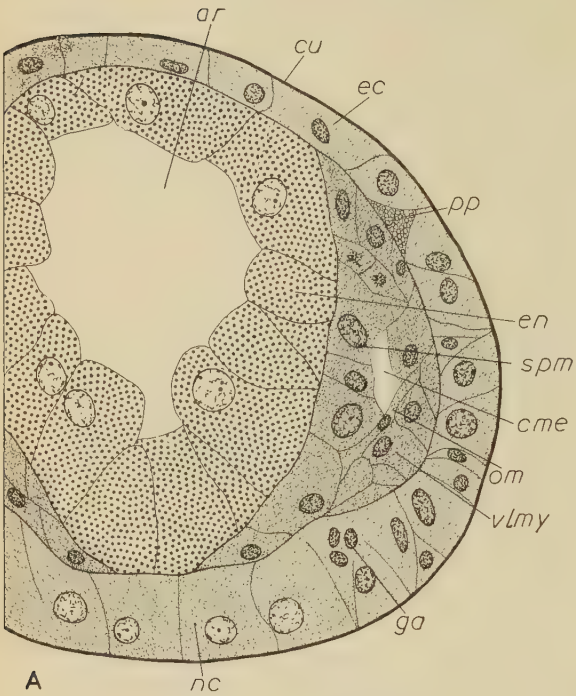
he ectoderm of the embryonic trunk. From the initially yolk-filled, un-erentiated ectoderm of the embryonic trunk segments arises the epithelium he segments, the cells of the metatroch and gastrotrochs, the chaetal sacs dorsal cirri, and the ventral nerve-cord. The cells of the neurotroch have ndependent origin, as already described (p. 104), but can be considered onjunction with the ectoderm.

)ver the outer surface of the segments the ectoderm cells secrete a thin cle, through which the cilia and later the chaetae project. Below it the eral epithelium of the segments remains one cell thick and the major nges during embryonic development are loss of yolk by the cells and the erentiation of some of them into scattered unicellular glands. By the end metamorphosis the epithelium of all the embryonic segments is fully erentiated.

he cells of the metatroch develop from ectodermal cells of the metatrochal ment (*me*, figs. 5, C; 11, A) and do not have an independent origin like those he prototroch and neurotroch. They begin to differentiate soon after the derm of the segment has been laid down, as a ring of 6 elongate cells nd the circumference of the segment, with a ventral and sometimes a ll dorsal gap. During the fourth day they become transformed into typical ted cells with large oval nuclei, granular outer cytoplasm, and a residuum olk globules internally. The numerous cilia are short at first but increase ngth to a maximum of 40μ by the seventh day (*mec*, fig. 13, A). Otherwise cells remain unchanged until the ninth day (fig. 13, B). They then begin egenerate and 2 days after hatching they have been completely resorbed.

he gastrotroch cells of the first three chaetigers develop in the same way he metatroch cells. They comprise a transverse row of four cells in each ment, two on each side. Those of the first segment begin to differentiate he fourth day (*gc*, fig. 17, B), and the others follow them successively. By end of the sixth day all are typical ciliated cells. They persist until the h day and are then resorbed.

he mid-ventral row of neurotroch cells is 4 to 5 cells wide anteriorly ig. 17, A), but only two cells wide over most of its length (*n*, figs. 9, B; A; 17, B–D). The cells are initially yolk-filled, but differentiate rapidly, oming wide transversely and narrow antero-posteriorly and inter-digitat-in the median line. They have the usual granular outer and yolky inner plasm, and large clear nuclei with two nucleoli. The numerous cilia, just in fig. 10, reach a maximum length of 40μ (figs. 13, A; 17, C). The cells begin to degenerate, narrowing transversely (with a consequent lessening idth of the neurotroch, p. 97) and being replaced by the developing tral nerve-cord. Neurotroch cells continue to be added posteriorly until ighth day, when the ectoderm of the last embryonic segment is laid down; once degeneration of the neurotroch has begun the newly formed cells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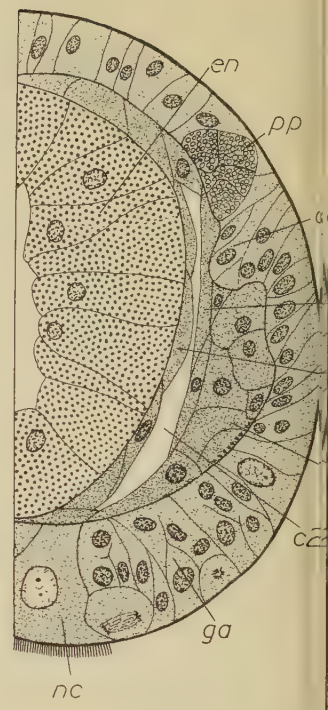
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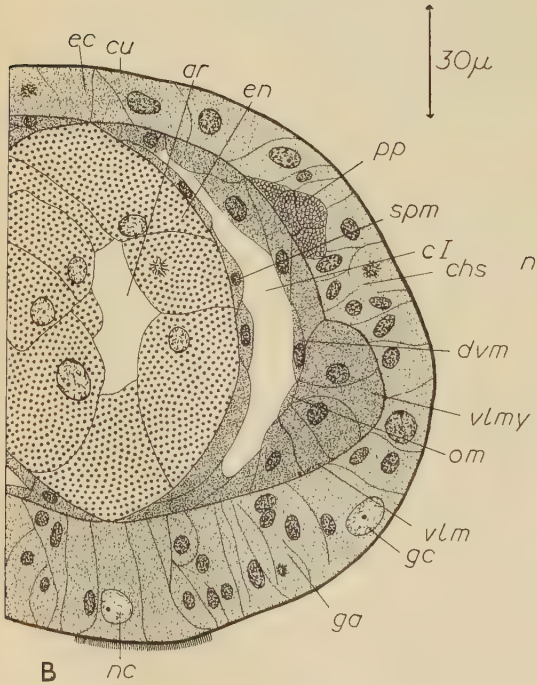
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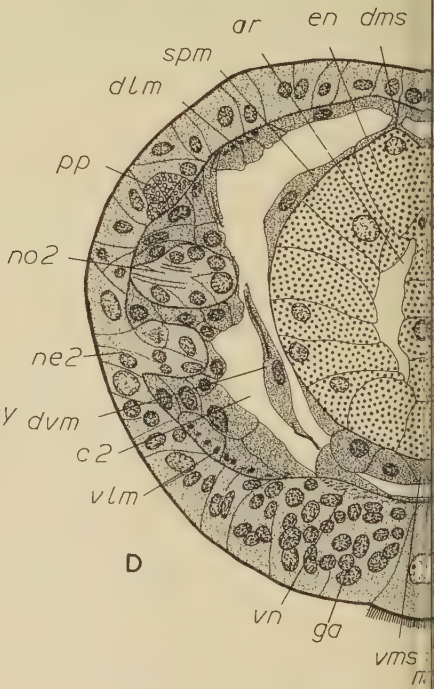
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ever reach a stage of differentiation more advanced than that of the degenerating cells immediately anterior to them. By the end of the twelfth day the eurotroch cells have been entirely resorbed.

The chaetal sacs originate as simple ingrowths of the lateral ectoderm figs. 17, C, D; 19, A, B). Beginning in the second trunk segment early on the ourth day (*chs*, fig. 17, B), they develop evenly from before backwards as ar as chaetiger 10. The first rudiments of the sacs are visible in each case efore the segment has been externally demarcated but after its mesodermal omites have begun to differentiate. In chaetiger 11, chaetal sac formation is onsiderably delayed and does not start until the fourteenth day. The chaetae re secreted by the cells of the sacs. The orientation of the sacs and development of the chaetae have already been described (pp. 101–2).

The notopodial cirri originate as solid outgrowths of the ectoderm and have o mesodermal components. The dorsal branchiae of chaetigers 10 and 11 lso begin to develop as solid ectodermal protuberances, but as they elongate hollow diverticulum of the mesoderm grows out into each one, forming a in endothelium below the ectoderm (*bme*, fig. 19, D). The branchial cilia are orne on a band of differentiated ectoderm cells.

The ventral nerve-cord arises from the ventral ectoderm on each side of the eurotroch. Radial division begins at the end of the third day in the cells mmediately adjacent to the neurotroch on the metatrochal segment (*ga*, g. 17, A) and spreads both posteriorly into successive segments (*vnc*, *ga*, gs. 9, C, D; 12, A; 14, A; 15, B) and laterally across individual segments. In he segments in which the ciliated cells of the metatroch and gastrotrochs ccupy part of the ventral surface, the ganglion cells develop round them and eplace them as the ciliated cells are resorbed.

The first 11 ganglia of the ventral nerve-cord (metatrochal segment to haetiger 10) are formed evenly from before backwards, the development of ach beginning at approximately the same time as that of the chaetal sacs. At rst the ganglia of successive segments are not distinguishable from each ther, but radial division ceases much earlier in the intersegments than in the anglia, so that the former become demarcated as transverse bands of relatively thin interganglionic ectoderm.

The neuropile first appears in the metatrochal ganglion on the seventh day *n*, fig. 13, A), on each side of the neurotroch (*n*). By the time of hatching it continuous as far back as chaetiger 9 (*vn*, figs. 12; 15, B), linking the ganglia

FIG. 17. A, transverse section through metatrochal segment of embryo of fig. 5, A. B, transverse section through chaetiger 1 of embryo of fig. 6, D, at level indicated. C, transverse section through chaetiger 2 of embryo of fig. 1, H, at level indicated. D, transverse section through chaetiger 2 of embryo of fig. 3, A, at level indicated.

ar, archenteron; *c 1*, *c 2*, coelomic cavity of chaetiger 1, 2; *chs*, chaetal sac; *cme*, coelomic vity of metatrochal segment; *cu*, cuticle; *dlm*, dorsal longitudinal muscle; *dms*, dorsal esentery; *dvm*, dorso-ventral muscle; *ec*, ectoderm; *en*, endoderm; *ga*, ventral nerve ganglion; *gc*, gastrotroch cell; *mlm*, median longitudinal muscle; *nc*, neurotroch cell; *ne 2*, neuropodium of chaetiger 2; *no 2*, notopodium of chaetiger 2; *om*, oblique muscle; *pp*, prototrochal ocesses; *spm*, splanchnic mesoderm; *vlm*, ventral longitudinal muscle; *vlmy*, myoblasts of ntral longitudinal muscle; *vms*, ventral mesentery; *vn*, ventral neuropile.

longitudinally across the intersegments and also fusing in the mid-line abov the degenerating neurotroch cells (fig. 19, B). Soon after the formation of it neuropile, each ganglion, which now consists of many cells, begins to becom more compact (*ga*, fig. 17, C, D; compare cerebral ganglion, p. 115) and to sin inwards, its lateral edges migrating towards the mid-line (fig. 19, B). With th final disappearance of the neurotroch the two halves of the nerve-cord fus mid-ventrally (*ga 3*, fig. 19, E).

Associated with the developing ganglia are a number of ectoderm cell which differentiate into unicellular glands (*ug*, figs. 12, B; 15, C). These ar later resorbed.

It is probably significant that at the time of hatching co-ordinated locc motory peristalsis occurs from the anterior end to chaetiger 9, when for th first time a continuous neuropile is present throughout these segments and i linked by circumpharyngeal commissures with the cerebral ganglion; but th limitations of technique made it impossible to determine whether any con nexion exists between the nervous and muscular systems at this time.

The neuropile extends into chaetiger 10 on the eleventh day (fig. 14, A). I chaetiger 11, however, active radial division in the ventral ectoderm does nc begin until the fourteenth day (compare chaetal sacs).

In the 5 days between hatching and the end of metamorphosis the gangli and neuropile become much better defined. At the anterior end of the trun the ganglia of the metatrochal segment and chaetiger 1 fuse to form th suboesophageal ganglion (*gas*, fig. 15, B, C), which receives the circumpharyn geal commissures. By the end of metamorphosis this ganglion and the larg ganglia of the second and third thoracic segments have sunk a considerab way into the body and are beginning to separate off from the ventral ectoderm The more posterior ganglia are still superficial and progressively less de veloped.

The endoderm. After 8 days of development continuity is establishe between the endodermal tube and the stomodaeum and proctodaeal p (p. 121 and p. 139; figs. 7, C; 12). As the stomodaeum continues to grow bacl wards the narrow anterior part of the endodermal tube is carried posterior into chaetiger 2 (fig. 14, A). Rapid cell-division in the remainder of th endoderm results in swellings (*ensw*) in the endodermal tube in chaetigers and 4 and convolutions posteriorly (fig. 8, A). The cells are still yolk-filled an undifferentiated when hatching takes place, but the anus, formed by a sma ectodermal intucking at the proctodaeal pit, opens for the first time at th stage (*a*, fig. 9, A).

Differentiation of the endoderm cells begins on the day after hatchin when the first cilia appear on the oesophageal epithelium, partly stomodae and partly endodermal in origin (p. 121; *oes*, figs. 14, A; 15, C). The distinctio between the two parts of the oesophagus disappears as the cells lose their yo contents. By the end of metamorphosis the cells of the oesophageal epitheliu are small and yolk-free, and their cilia very prominent (*oes*, figs. 8, A; 15, 19, F).

Throughout the rest of the gut the epithelium is wholly endodermal in origin. In chaetiger 3 a pair of small lateral diverticula of the endoderm appears on the fourteenth day (*gd 1*, fig. 15, B), the rudiments of the diverticula of the adult gut. In chaetigers 4 to 6 three segmental swellings of the gut are developed, and here the cells become covered with fine cilia while still full of yolk (*sto*, fig. 15, B). Where the endodermal tube is convoluted the yolk disappears more rapidly and only scattered cilia are produced. On the fourteenth day (fig. 15, B) faint peristaltic movements are seen for the first time in this part of the gut.

By the end of metamorphosis the gut is functional (fig. 8, B). The cells are devoid of yolk and the gut-lumen contains diatomaceous food material (*f*). The oesophagus (*oes*) extends into chaetiger 4, where it meets the stomach (*sto*), derived from the original segmental swellings. Ventrolaterally at the junction of the oesophagus and stomach arise the two gut diverticula (*gd 1*, *gd 2*), each lined by pale-staining cells with large nuclei. The stomach occupies most of chaetigers 4 to 6 as a capacious sac lined by a thin epithelium of dark-staining cells with small nuclei, covered with short fine cilia (*sto*, fig. 19, E). The convoluted region of the endodermal tube now forms the intestine (*int*), with a cuboidal epithelium and scattered cilia. At the posterior end is a short, straight, ciliated rectum (*re*). Both the intestine and the rectum show active peristalsis resulting in defaecation.

The mesoderm of the embryonic trunk. The mesoderm of the embryonic trunk segments gives rise to the coelomic peritoneum, the trunk musculature, and the walls of the blood-vessels. The origin of its somatic, splanchnic, septal, and mesenterial elements has already been described (p. 108). It differentiates evenly from before backwards as far as chaetiger 10. Chaetiger 11, in this as in other respects, has a delayed development. By the end of metamorphosis all the yolk in the mesoderm is resorbed.

The splanchnic mesoderm forms a single layer of cells covering the gut and proboscis apparatus (*spm*, figs. 13, A; 17, A–C). The cells gradually become attenuated as their yolk contents disappear (figs. 15, C; 17, D; 19, B) and in the metamorphosed larva only their nuclei are conspicuous (*spm*, fig. 19, E, F). Where the splanchnic mesoderm forms part of the wall of the proboscis cavity (p. 123) the cells are initially cuboidal (fig. 13, A; *spm*, fig. 14, D), but later become flat.

By the end of metamorphosis the intestine and rectum both show peristaltic activity, but no trace of musculature has been found in the splanchnic mesoderm, even with the highest magnifications. Either the mesoderm cells are themselves contractile or they contain muscle fibrils which are extremely thin.

The intersegmental septa vary in their developmental history. The breakdown of the septum between the metatrochal segment and the mouth region on the eighth day has already been discussed (p. 125). Before hatching, the septum between the metatrochal segment and chaetiger 1 disappears so that a continuous coelomic cavity occupies the prostomium, the mouth region, the

metatrochal segment, and chaetiger 1 (*hc*, fig. 8, A). This is the adult hea coelom, bounded posteriorly by the septum between chaetigers 1 and 2 (*is 1* fig. 15, C). By the end of metamorphosis the latter forms a thin double epi thelial wall. In the septum between chaetigers 2 and 3, on the other hanc many of the cells of the anterior epithelial layer give rise to the dorso-ventra muscle-fibres (*ism*, fig. 19, C) characteristic of adult thoracic segments. Th third and more posterior adult septa, like the first, form thin double epitheli walls between the segmental coeloms (*is 3*, *is 4*, *is 4*, fig. 19, C, E).

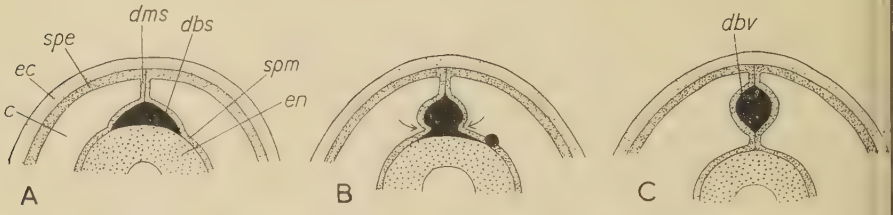


FIG. 18. Development of the dorsal blood vessel in *S. armiger*, diagrammatic transvers section (not to scale).

c, coelom; *dbs*, dorsal blood sinus; *dbv*, dorsal blood-vessel; *dms*, dorsal mesentery; *ec*, ectc derm; *en*, endoderm; *spe*, somatic peritoneum; *spm*, splanchnic mesoderm.

The later development of the dorsal and ventral mesenteries, both of whic persist in the embryonic trunk segments, is greatly influenced by the forma tion of the main longitudinal blood-vessels. The dorsal longitudinal vesse begins to develop on the eighth day. In the dorsal mid-line the splanchni mesoderm separates from the endoderm both in the metatrochal segment an at the posterior end of the body, to give blood sinuses (*dbs*, fig. 12, A). Separa tion of the two layers spreads backwards and forwards as differentiation of th gut proceeds (*dbs*, figs. 14, A; 15, C), and at the same time the lateral edges c the mesodermal wall of the sinus come together in the dorsal mid-line abov the gut to give the dorsal blood-vessel and complete the dorsal mesente (*dbv*, figs. 15, C; 18; 19, B).

The ventral longitudinal vessel develops in a different way, by separatio of the two epithelia of the already established mesentery. Beginning at th anterior and posterior ends of the embryo at about the hatching stage, th separation passes towards the middle of the embryo (*vbv*, figs. 14, A; 19, E and is completed before the end of metamorphosis (*vbv*, fig. 15, B). The wall of the ventral vessel in the metamorphosed larva have the same form as thos of the dorsal vessel, except below the oesophagus where they are more tha one cell thick (*vbv*, fig. 19, B, F).

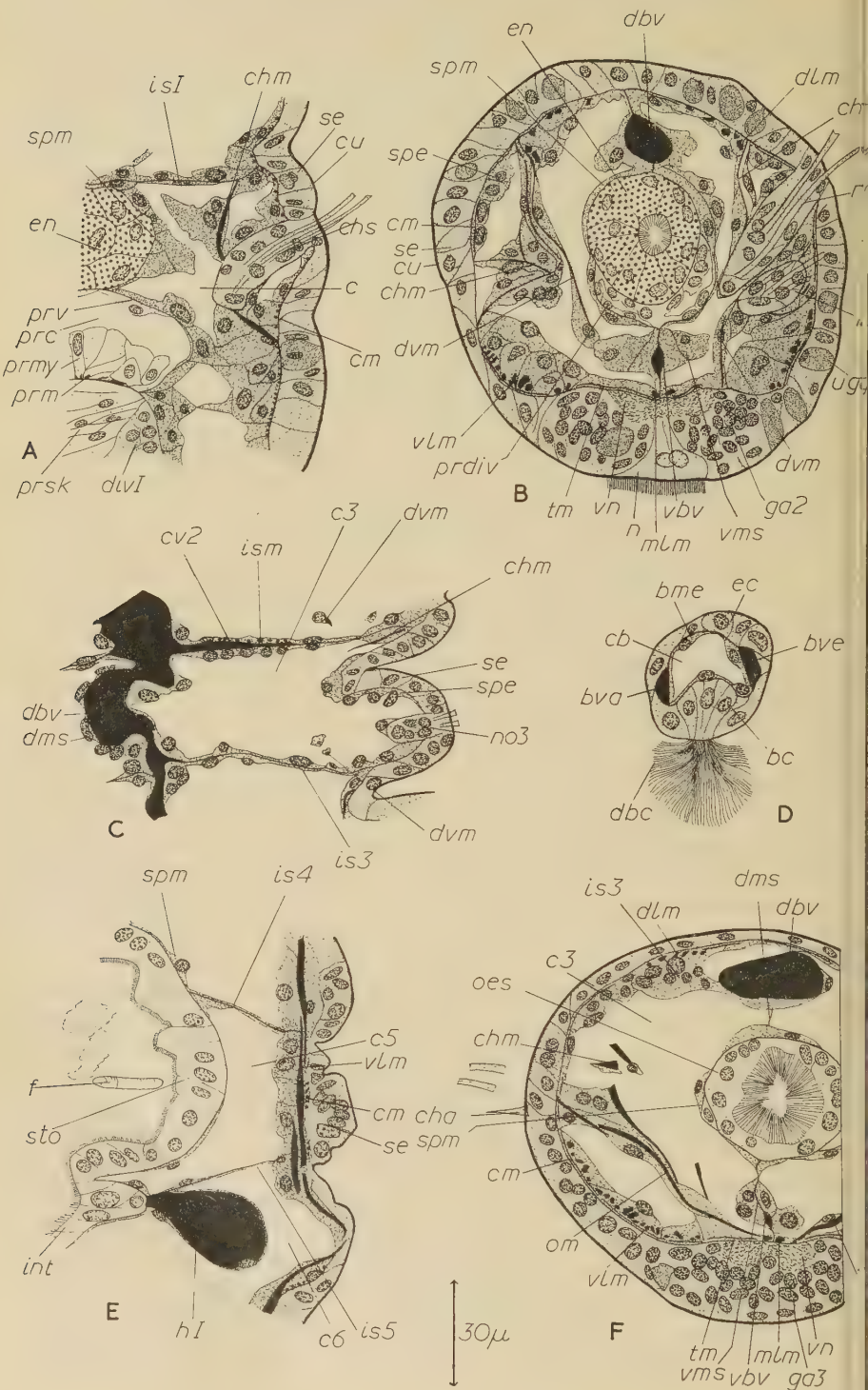
The dorsal and ventral longitudinal vessels are connected posteriorly by blood sinus between the endoderm and the residual mesoderm (*bs*, fig. 9, A). At the anterior end of the embryo a more complex condition arises (fig. 15, A). In the mouth region, where the dorsal mesentery does not persist, the dorsa blood-vessel is formed by separation of the somatic mesoderm from th ectoderm in the dorsal mid-line (*dbv*, fig. 14, C). At the level of the prostomia

nnulus the separation extends on each side down the inner surface of the erebral ganglion along the line of the septa between ca_0 and ca_1 (*prov*, igs. 5, B; 14, B) and then turns medially to meet in the ventral mid-line below he cerebral neuropile. The prostomial vessels so formed are cut off from the ctoderm in the same way as that in which the dorsal longitudinal vessel is cut ff from the endoderm. At the same time a pair of circumpharyngeal vessels s formed, partly by cells associated with the anterior ends of the anterior blique muscles (i.e. cells derived from the septa between ca_0 and ca_1), partly y separation of the splanchnic mesoderm from the lateral wall of the pharynx nd proboscis skeleton (*cphv*, fig. 14, C), and partly by a space formed between he splanchnic mesoderm and the inner layer of the wall of the proboscis avity (*cphv*, fig. 14, D). The two vessels meet posteriorly in a median sinus *pras*, fig. 15, A), which receives the anterior end of the ventral longitudinal essel (*vbv*).

Towards the end of metamorphosis the anterior commissural vessels begin o develop by separation of the two epithelia in the intersegmental septa (*cv 2*, g. 19, C). The dorsal ends of the first two (*cv 1*, *cv 2*) are seen in fig. 15, B. t the same time an afferent branchial vessel appears on each side in the eptum between chaetigers 10 and 11, running from the ventral longitudinal essel to the posterior side of the base of one of the dorsal branchiae of haetiger 10 (*av*, fig. 15, B). Within the branchia it continues as an afferent ntra-branchial vessel (*bva*, fig. 19, D) formed by separation of the somatic nesodermal lining (*bme*) of the branchia from the ectoderm. On the anterior ace of the branchia an afferent intra-branchial vessel arises in the same way *ve*, fig. 19, D) and the two are connected by similar smaller vessels running ransversely round the branchia. A short efferent branchial vessel formed in ne septum between chaetigers 9 and 10 then connects the efferent intra-ranchial vessel with the dorsal longitudinal vessel. By the end of meta-norphosis a similar blood system is developing in association with the ranchiae of chaetiger 11. In relation to the antero-posterior mid-dorsal water urrent, the arrangement of the vessels conforms with the counter-current rinciple.

On the fourteenth day the first pair of lateral hearts begins to develop. Just ehind the intersegmental septum between chaetigers 5 and 6 the splanchnic esoderm on each side lifts from the endoderm, forming a large thin-walled ac (*h 1*, fig. 19, C). The sacs elongate dorso-ventrally and separate from the ut except at the ventral end. Soon after metamorphosis a lateral blood-vessel formed on each side of the gut by separation of the splanchnic mesoderm om the endoderm at the level of the lower ends of the lateral hearts. The pper ends of the hearts eventually become connected with the commissural essels in the septum immediately anterior to them.

All the blood-vessels in *S. armiger* arise as haemocoelic (blastocoelic) spaces y separation of mesoderm from mesoderm, endoderm, ectoderm, and pro-oscis apparatus cells. The walls of the vessels are simple epithelia overlying basement membrane.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The somatic mesoderm gives rise to the trunk musculature, differentiated evenly from before backwards, and to the somatic peritoneum. The earliest visibly differentiated cells within it are the myoblasts of the ventral longitudinal muscles (*vlmy*, fig. 17, A), a continuous ventro-lateral band of large cells in the unsegmented mesodermal bands on each side. They remain unaffected by segmentation and the muscles formed in them extend across the intersegmental boundaries. The cells elongate and a number of very thin muscle-fibres appears in each, closely apposed to the ectoderm (*vlm*, fig. 17, B, C). The first fibres are seen late on the fourth day, in the prototrochal region (p. 94), metatrochal segment, and chaetiger 1, when the embryo begins to show contractile movements. They then appear in successive segments until at hatching they are present to chaetiger 9, the last segment undergoing active peristalsis at this stage. At the same time the fibres thicken and increase in number (*vlm*, figs. 13, A; 17, D) and those of successive cells run together to form long muscle-strands extending over several segments (*vlm*, figs. 13, A; 16). After hatching the myoblasts gradually lose their yolk contents and become smaller, but their cytoplasm persists as part of the somatic peritoneum (*vlm*, figs. 19, B, F). The ventral longitudinal muscles appear in chaetiger 10 just after hatching, but those of chaetiger 11 are not formed until the fourteenth day.

The dorsal longitudinal muscles arise in the same way as the ventral, but a little later in each segment. Their myoblasts are not as conspicuous as the ventral myoblasts (*dlm*, fig. 17, C, D: 19, A, B).

Part of the retractor muscle system of the lower lip (p. 127; fig. 16) is formed from segmental somatic mesoderm. The median longitudinal muscles (*mlm*) develop at the same time as the ventral longitudinal muscles in the cells on each side of the ventral mesentery and extend through the embryonic trunk segments (figs. 14, D; 17, D; 19, B). The posterior ends of the longitudinal

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FIG. 19. A, frontal section through chaetiger 1 of embryo of fig. 3, C. B, transverse section through chaetiger 2 of embryo of fig. 14, A, at level indicated. C, frontal section through dorsal blood-vessel of pre-adult of fig. 8, B. D, transverse section through dorsal branchia of chaetiger 10 of pre-adult of fig. 8, B. E, frontal section through developing first lateral heart of pre-adult of fig. 8, B. F, transverse section through chaetiger 3 of 18-day pre-adult.

bc, branchial ciliated cells; *bme*, branchial mesoderm; *bva*, afferent intrabranchial blood-vessel; *bve*, efferent intrabranchial blood-vessel; *c*, coelom; *c 3*, *c 5*, *c 6*, coelomic cavities of chaetigers 3, 5, 6; *cb*, branchial coelom; *cha*, chaeta; *chm*, chaetal sac muscle; *chs*, chaetal sac; *cm*, circular muscle; *cu*, cuticle; *cv 2*, commissural blood-vessel of chaetiger 2; *dbc*, branchial cilia; *dbv*, dorsal blood-vessel; *div 1*, first pharyngeal diverticulum; *dlm*, dorsal longitudinal muscle; *dms*, dorsal mesentery; *dvm*, dorso-ventral muscle; *ec*, ectoderm; *en*, endoderm; *f*, food; *ga 2*, *ga 3*, ventral ganglia of chaetigers 2, 3; *hl*, first lateral heart; *int*, intestine; *ism*, dorso-ventral septal muscle; *cs 1*, *3*, &c., intersegmental septa behind chaetigers 1, 3, &c.; *mlm*, median longitudinal muscle; *n*, neurotroch; *ne 2*, neuropodium of chaetiger 2; *no 2*, *no 3*, notopodium of chaetiger 2, 3; *oes*, oesophagus; *om*, oblique muscle; *prc*, proboscis cavity; *prdiv*, diverticulum of proboscis cavity; *prm*, proboscis retractor muscles; *prmy*, myoblasts of proboscis retractor muscles; *prsk*, proboscis skeleton; *prv*, ventral wall of proboscis cavity; *se*, segmental ectoderm; *spe*, somatic peritoneum; *spm*, splanchnic mesoderm; *sto*, stomach; *tm*, transverse muscle; *vbv*, ventral blood-vessel; *vlm*, ventral longitudinal muscle; *vms*, ventral mesentery; *vn*, neuropile of ventral nerve-cord; *ug*, unicellular gland.

retractors also differentiate from somatic mesoderm cells in the metatrocha segment (*lrm*, fig. 14, D).

The development of circular muscles in the embryonic trunk segments is very difficult to study, owing to their thinness and their close proximity to the ectoderm. They arise in the somatic mesoderm as a single layer of fibres lying just internal to the ectoderm (*cm*, figs. 13, A; 19, A, B). They appear to be differentiated initially in the lateral somatic mesoderm cells and then to grow dorsally and ventrally between the longitudinal muscles and the ectoderm, meeting in the dorsal mid-line and extending to each side of the developing ventral nerve-cord (*cm*, fig. 19, F). After metamorphosis, when the ventral nerve-cord sinks inwards, the circular muscle-fibres penetrate between it and the ectoderm to meet in the ventral mid-line. Like the longitudinal muscles the circular muscles differentiate in the metatrochal segment on the fourth day and then successively in more posterior segments. At hatching they are functional in chaetiger 9. They appear in chaetiger 10 shortly after hatching but in chaetiger 11 not until the end of metamorphosis.

A number of thin transverse muscle-fibres are simultaneously developed in the somatic mesoderm covering the inner surface of the ventral nerve-cord (*tm*, figs. 12, B; 13, A; 15, C; 16; 19, B); also a series of oblique and dorso-ventral muscles, the details of which vary in different segments. Oblique fibres running from the lateral margin of the ventral longitudinal muscles to the lateral margin of the neuropile on each side, occur in every segment just in front of its posterior septum. They are differentiated in cells which lie at first on the coelomic surface of the ventral longitudinal myoblasts (*om*, figs. 4, C; 5, C). As the muscles develop and the yolk is lost from the mesoderm cells, the oblique muscles separate from the ventral longitudinal muscles to run freely across the coelom (*om*, fig. 19, F). In the metatrochal and thoracic segments a second series of oblique muscles is formed in the same way, running from the dorso-lateral body-wall to the mid-line of the developing neuropile, and three dorso-ventral muscle-fibres develop on each side from cells which separate from the somatic mesoderm as the coelom expands (*dvm*, figs. 17, B–D; 19, B, C). Each dorso-ventral fibre forms in three or four elongate cells which run together at their ends. The same process is described in *Aricia foetida* by Schaxel (1912). The oblique muscles of chaetiger 10 are well formed by the time of hatching but those of chaetiger 11 are just beginning to develop by the end of metamorphosis.

Each notopodial and neuropodial chaetal sac has its own set of muscles. Mesoderm cells attached to the ingrowing sac rudiment and to the ectoderm elongate as the rudiment grows, and a single muscle-fibre differentiates in each (*chm*, fig. 19, A, B). In this way two cones of muscle-fibres are formed, one attached to the inner end of the chaetal sac and one half-way along it (fig. 8, A, B). The muscles develop with the sacs, evenly from before backwards. Fibres form in the myoblasts in chaetiger 1 on the ninth day. By the end of metamorphosis only those of chaetiger 11 are not functional.

Thus the embryonic trunk musculature in *S. armiger* is formed from the

omatic mesoderm. It comprises the dorsal and ventral longitudinal muscles, he circular muscles, the transverse, oblique, and dorso-ventral muscles and lso the chaetal sac muscles and the retractors of the lower lip. At the time of atching every segment but chaetiger 11 already has a well-developed muscu- ature (compare p. 97), although the segments are largely undifferentiated n other respects.

The pygidium. In the trochophore the pygidial ectoderm forms a simple cap f yolk-filled cells covering the posterior end of the embryo (p. 105; *pye*, g. 4, A–C). The distinction between the pygidium and the ectoteloblasts (*et*) s emphasized during the third day by the differentiation, from cells lying mmediately posterior to the teloblasts, of the telotroch cells (*t*, fig. 5, C). There are four of these, two latero-dorsal and two latero-ventral, forming a ransverse ring of cells complete except for a small mid-ventral gap (*tc*, g. 20, B). When cilia first begin to appear on the cells they are fully differen- iated, with large oval nuclei and granular outer cytoplasm. The cilia grow apidly to a length of about 40μ, but otherwise the cells remain unchanged ntil the ninth day. By the eleventh day they have been resorbed.

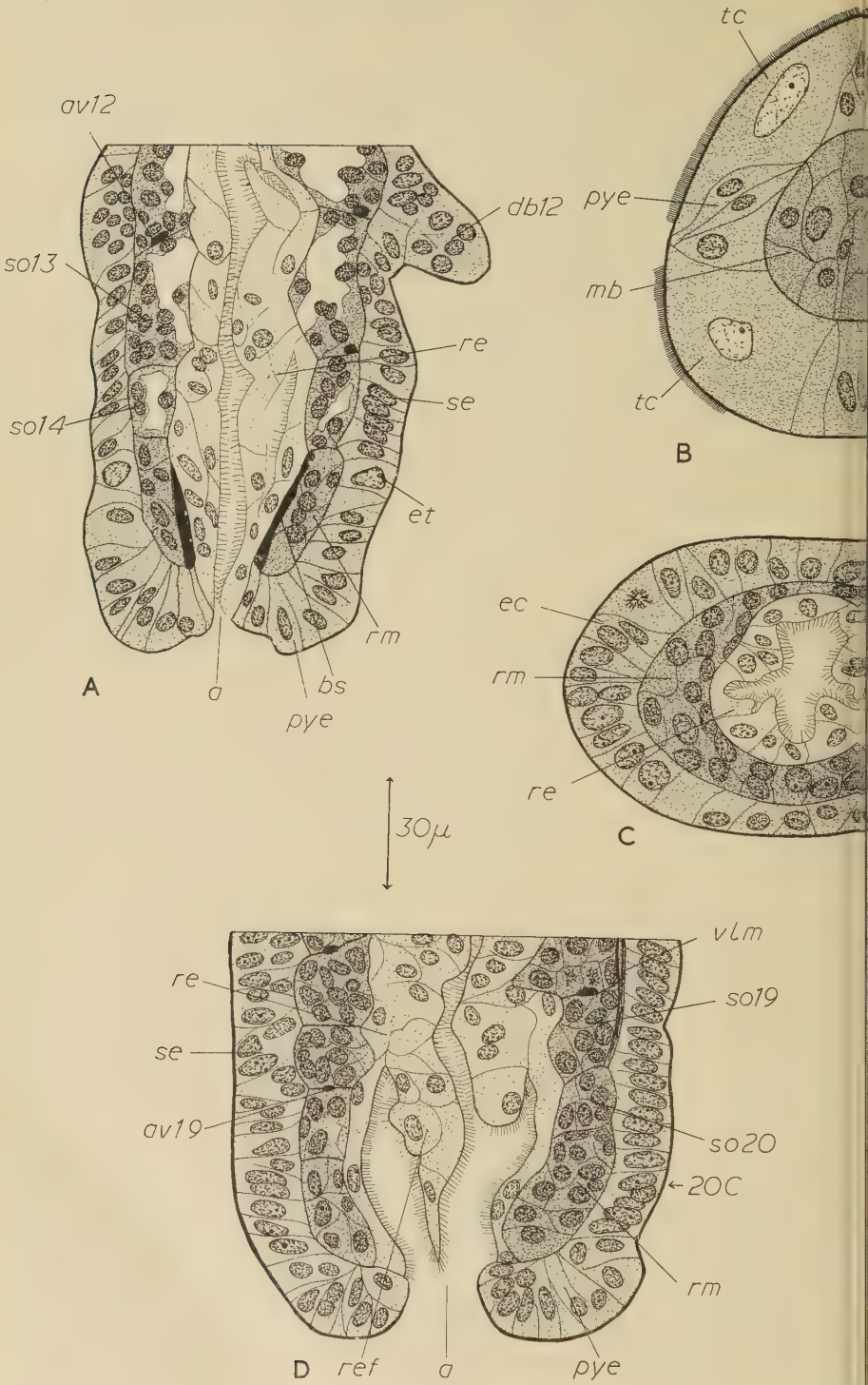
The pygidial ectoderm undergoes very little change until the eighth day, hen coincident with the back-growth of the endoderm a small invagination f the ectoderm occurs at the proctodaeal pit (*a*, fig. 7, C, D). By the tenth day he ectoderm and endoderm have become confluent around the now open nus (*a*, fig. 9, A) and the yolk is disappearing from the ectoderm cells.

During metamorphosis the residual mesoderm (*rm*) undergoes no change, hile the endodermal tube which it surrounds differentiates into the posterior nd of the rectum (*re*). The major changes in the pygidium between the time f formation of the anus and the end of metamorphosis take place in the ctoderm. With the resorption of the telotroch the pygidial ectoderm is eparated from the ectoderm, of chaetiger 11 only by the dormant ectodermal rowth zone.

On the eleventh day the ventral ectoderm of the pygidium begins to grow, esulting in migration of the anus to a postero-dorsal position by the end of netamorphosis (fig. 15, B). The lateral protuberances of the pygidium which levelop during this period (*py*, fig. 8, B) are formed by thickening of the ctoderm, which becomes several cells deep. The anal cirri (*ac*) are solid out- rowths of the same ectoderm. By the end of metamorphosis all the cells of he pygidial region (ectoderm, endoderm, and mesoderm) are devoid of yolk.

Formation of the post-embryonic segments

With the completion of metamorphosis the embryo becomes a fully differen- iated pre-adult and the pre-pygidial growth zone once more becomes active p. 100; fig. 2). Each of the post-embryonic abdominal segments produced by is formed in the same way. Ectoderm and mesoderm are budded off nteriorly from the ectoteloblast ring (*et*) and residual mesoderm (*rm*) (fig. 20, , C, D), and at the same time the gut increases in length. After 2, later 3, days, pair of lateral coelomic cavities appear in the new mesoderm and the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osterior margins of a new pair of somites become delimited. Ectoderm forma-on is completed after somite formation, as in the embryonic segments, and the egment is not externally demarcated for another 2, later 3 or 4 days (figs. 3, ; 6, B; 20, A, D). At this stage the segment has a simple ectodermal epithelium nd somatic, splanchnic, septal, and mesenterial mesoderm, very little ifferentiated.

The delineated segment rapidly attains a functional condition (p. 100). The ctoderm gives rise to the epidermis, chaetal sacs, dorsal cirri, and ventral erve ganglion. The somatic mesoderm forms peritoneum, dorsal and ventral ngitudinal muscles, oblique muscles, and circular muscles; the splanchnic esoderm forms a thin peritoneum over the rectum. Dorsal and ventral ngitudinal blood-vessels and afferent and efferent branchial vessels develop n the double epithelial mesenteries and septa. The dorsal branchiae are dentical with those of chaetigers 10 and 11. All the organs in the post-mbryonic segments are formed in the same manner as their counterparts in ne embryonic segments.

urther development of the pre-adult

As the number of body segments increases the different regions of the gut ove progressively backwards. The oesophagus lengthens and at the end of weeks (compare fig. 6, B) occupies chaetigers 2 to 5. In chaetiger 5 it meets ne stomach, which now extends to the posterior end of chaetiger 7 (compare g. 8, B and p. 133). The convoluted intestine runs back to chaetiger 17, and followed by the rectum (*re*), opening at the anus (*a*, fig. 20, D). The lateral ut diverticula now lie in chaetigers 4 and 5 (compare fig. 8, B, chaetigers 3 nd 4). It has been impossible to decide how far these changes result from rowth of the various gut regions and their displacement relative to the esoderm (e.g. the oesophagus pushing the anterior end of the stomach ackwards through the splanchnic peritoneal tube, the stomach pushing the nterior end of intestine owing to oesophageal and to its own growth, and o on) and how far from the redifferentiation of one type of gut cell into nother (e.g. stomach into oesophagus).

No trace of nephridia has been found in developmental stages up to 6 weeks ld. In the adults nephridia occur only in the genital segments, chaetiger 20 nwards (Mau, 1882), and these are not present in the early pre-adult.

In the mesoderm and ectoderm of the pre-adult no major changes occur uring the first 6 weeks other than increase in bulk (particularly of the muscu-ature), formation of dorso-ventral muscles in the intersegmental septa between

FIG. 20. A, frontal section through posterior end of pre-adult of fig. 3, E. B, transverse ection through telotroch of embryo of fig. 1, F. C, transverse section through growth zone f pre-adult of fig. 6, B. D, frontal section through posterior end of pre-adult of fig. 6, B. *a*, anus; *av 12*, *av 19*, afferent branchial blood vessel of chaetiger 12, 19; *bs*, blood sinus; *b 12*, dorsal branchia of chaetiger 12; *ec*, ectoderm; *et*, ectoteloblast; *mb*, mesodermal band; *ve*, pygidial ectoderm; *re*, rectum; *ref*, rectal fold; *rm*, residual mesoderm; *se*, segmental ctoderm; *so 13*, *so 14*, *so 19*, *so 20*, mesodermal somites of chaetigers 13, 14, 19, 20; *tc*, lotroch cell; *vlm*, ventral longitudinal muscle.

chaetigers 3 and 4, and 4 and 5, and the completion of the segmental com misural blood-vessels. The proboscis apparatus also increases in size an develops a number of additional non-mesodermal muscles. After 6 weeks however, when the segments behind chaetiger 3 begin to be transformed int thoracic segments (p. 102), the coeloms of chaetigers 2 to 5, which contain th oesophagus, become filled with a parenchymatous tissue derived from th peritoneum. In this the adult thoracic dorso-ventral musculature, not repre sented in the early pre-adult, is formed. A similar parenchyma occurs i *A. foetida* (Salensky, 1883). As the oesophagus continues to grow posteriorl and the number of thoracic segments increases, both parenchymal and septa dorso-ventral muscles are found in more and more posterior segments. I other respects the musculature of the adult trunk conforms to the basic patter laid down in the embryo.

When the pre-adult is about 6 months old the adult proboscis is differen tiated as a series of lobes of the oesophagus in the middle region of the thorax Associated with it is an eversion musculature (see Mau, 1882), developed i the surrounding mesodermal parenchyma. The pre-adult proboscis apparatu and the mesodermal muscles attaching it to the body-wall then disappea completely, leaving a simple pharynx leading into the ciliated oesophagus At the same time the mouth region and metatrochal segment of the larva fus to form the adult peristomium. *A. foetida* undergoes similar changes (Eisig 1914).

The adult head coelom occupies the prostomium, peristomium, an chaetiger 1 (see p. 133). The ventral ganglion of chaetiger 1 is formed by fusion of its own ganglion with that of the metatrochal segment, and the only ele ments of the central nervous system in the peristomium are the circum pharyngeal commissures.

Investigation of the course of epigenesis in *S. armiger* has thus yielded muc detailed information. Relating as it does the structure of the adult to its pre sumptive organization in the trochophore, it places our understanding o polychaete embryology in a new perspective. For the first time it has prove possible to show that the trochophore, which persists in a recognizable form even though yolky and non-pelagic, incorporates the rudiments of the futur trunk segments wholly within a prepygidial growth zone made up of 2c ectoteloblasts and 4d mesodermal teloblasts. The parts of the trochophor anterior and posterior to this, prototrochal region (= future prostomium an mouth region) and pygidium respectively, have a different developmenta history from the trunk segments. The latter, although formed in two succes sive series, embryonic (before metamorphosis) and post-embryonic (afte metamorphosis), are all identical in origin and development. Their ectoderm arising from the 2d ectoteloblast ring of the trochophore, forms segmenta epithelium, chaetal sacs, and ventral nerve ganglia. Their mesoderm, lai down initially as segmental pairs of hollow somites in the mesodermal band derived from the 4d teloblasts of the trochophore, gives rise to splanchnic an

omatic peritoneum, septa and mesenteries, trunk musculature, and the walls f blood-vessels. In the prototrochal region a different condition obtains. The d mesodermal bands invade this part of the body early in development, and here develop three pairs of lateral coelomic cavities and an anterior median avity. To the enclosure of the latter ectomesoderm of the third quartette nakes some contribution. The walls of these seven cavities subsequently form he mesoderm of the prostomium and mouth region together, and the division pparent between the ectodermal components of these two parts of the body s not reflected internally. Furthermore the ectoderm of the prototrochal egion, forming mainly epithelium, has a composite origin different from that f the trunk segments, and the prostomial ectoderm becomes distinct from hat of the mouth region only as a consequence of formation of the cerebral anglion within it. This ganglion develops independently of the ventral anglia of the trunk segments, and becomes united to them only later in levelopment, by a pair of circumpharyngeal commissures. There is no ventral anglion in the mouth region.

The gut, which opens anteriorly on to the mouth region and posteriorly n to the persistent post-segmental pygidium, develops from stomodaeum and ndoderm alone. The former gives rise to the pharynx and part of the oesophagus, the latter to the remainder of the gut. Associated with the stomodaeum is a proboscis apparatus, developed wholly from second and third uartette (ectomesoderm) cells, having ectomesodermal retractor muscles nd a non-coelomic internal cavity.

The anterior end of the body subsequently undergoes further change. The nterior median and three pairs of lateral coelomic cavities of the prototrochal egion fuse with each other and with the paired coelomic cavities of the first wo trunk segments to form the adult head coelom. The mouth region fuses ith the first trunk segment (metatrochal segment) to form the adult peristomium, and the ventral ganglion of the first segment fuses with that of the econd (chaetiger 1) to form the sub-oesophageal ganglion.

In the discussion which follows the points summarized above are considered in the context of the problems raised in the Introduction.

Discussion

he polychaete trochophore

It is well known that in all polychaetes the processes of spiral cleavage, astrulation, and blastoporal closure result in formation of a trochophore. leavage is determinate, and each constituent part of the trochophore takes rigin from a specific cleavage blastomere. The polychaete trochophore, however, shows great variation of form and habit. Concomitantly, although cleavage always follows an identical course up to the 64-cell stage, the products f the cleavage blastomeres are themselves variable (Treadwell, 1901; Delsman, 1916). In polychaetes with microlecithal eggs, such as *Eupomatus* = *Hydroides*) *uncinatus* (Shearer, 1911), with an egg 55μ in diameter, the ajority of the cleavage blastomeres give rise to differentiated larval tissue in a

pelagic planktotrophic trochophore and only a minority contribute to the rudiments of the future adult. In some species of this kind, such as *Owenia fusiformis* (D. P. Wilson, 1932) and *Lopadorhynchus* (Meyer, 1901), and also in *Polygordius* (Woltereck, 1902, 1904), the planktotrophic trochophore becomes very complex before the adult rudiments begin to develop, and the amount of presumptive adult tissue gained from the egg is very small indeed.

In species with yolky eggs, on the other hand, the opposite is found. Even when the egg is small and gives rise to a pelagic trochophore, e.g. *Neanthes succinea* (= *Nereis limbata*) (E. B. Wilson, 1892), a higher proportion of the cleavage blastomeres contains presumptive adult tissue, and larval organs other than the prototroch are not developed in the trochophore to the same degree as in microlecithal forms. When increased yolk content is also associated with an increase in egg-size, especially above 160μ in diameter (Newell, 1951), the functionally differentiated organs of the trochophore are eliminated almost completely from development, the larva is non-pelagic, and the cleavage blastomeres give rise almost entirely to adult rudiments. This is the case in *S. armiger*. Cleavage in this species results in the formation of relatively very large 2d and 4d cells. Since these cells are the teloblasts of the ectoderm and mesoderm of the adult trunk segments (pp. 106–11), it is possible for a number of trunk segments to be formed from material already contained in the egg without additional building material being acquired by the developing animal through active feeding. Differentiation to a miniature adult can therefore proceed directly, and in conformity with this the presumptive rudiments which in a microlecithal form would give rise to functional larval organs have been largely lost from the egg. The prototroch rudiment is retained, however, and the prototroch still shows precocious differentiation. Neurotroch and telotroch rudiments also occur, as in species with a pelagic planktotrophic trochophore, but their differentiation is delayed until a later stage of development.

In view of the great divergence between the lecithotrophic trochophore of *S. armiger* and the more characteristic pelagic planktotrophic trochophore product of a microlecithal egg in polychaetes, some appraisal of the possible evolutionary history of the polychaete trochophore appears to be required. There seems little doubt that the trochophore has always been a larval stage in polychaetes, since it can be recognized, usually in a modified form, in every species. Like many other kinds of larvae it has undergone its own evolutionary changes, and in interpreting these, three main factors must be taken into account. First, a very similar pelagic planktotrophic trochophore is found in several serpulids (Soulier, 1902; Shearer, 1911; Segrove, 1941), *Podarke obscura* (Treadwell, 1901), many polynoids (Gravely, 1909) and other polychaetes with microlecithal eggs, all in widely separated families. Secondly, a trochophore of the same type occurs in *Polygordius* (Woltereck, 1904), *Thalassema* (Torrey, 1902) and primitive molluscs, phylogenetically divergent from polychaetes but all showing spiral cleavage. Thirdly, whatever the effect of yolk and increased egg-size on the other larval organs of polychaetes, the

lastomeres 1a to 1d always give rise to 16 primary trochoblasts which ifferentiate precociously(*S. armiger* (this account, p. 104)), *Lepidonotus sp.* and *Iarmothoe sp.* (Mead, 1897), *Podarke obscura* (Treadwell, 1901), *Capitella apitata* (Eisig, 1898), *Arenicola cristata* (Lillie, 1905), *Clymenella torquata* and *Amphitrite ornata* (Mead, 1897) and *Protula meilhaci* (Soulier, 1920), in hich the trochophore varies from the pelagic planktotrophic form in *Protula* o the non-pelagic lecithotrophic form in *Scoloplos*). The probable interpretaon to be put on these facts is that a simple pelagic planktotrophic trochophore primitive to polychaetes and was inherited from the common ancestor of nnelids and molluscs. Subsequent evolution within the polychaetes has led, n the one hand, to elaboration of the trochophore as a feeding organism (e.g. *Owenia fusiformis*, Wilson, 1932), on the other, to reduction of the functional ochophore organs in favour of a yolky egg and a more direct development .g. *S. armiger*). A variety of intermediate conditions also occurs.

Segrove (1941) suggests that the uniformly ciliated larva which precedes ne trochophore in eunicids and some maldanids represents an ancestral olychaete larva, from which feeding trochophores and yolky larvae have risen by divergence. This interpretation appears to be based on a considera-on of too limited evidence. While it is a conclusion which could conceivably llow from a comparison of external features alone, one cannot ignore in this ntext the highly probable phylogenetic significance of the similarity of some olychaete and mollusc trochophores, and the existence of an actual proto-och in polychaetes with yolky eggs.

The relationship between the structure of the trochophore and the com-osition of the adult body in polychaetes has been variously interpreted. It is enerally accepted that segmentation in invertebrates originally evolved in the unk region of an elongate ancestor, with anterior and posterior unsegmented arts of the body persisting at each end. The majority of authors therefore ivide the trochophore into three regions:

(1) the apical plate, bounded posteriorly by the prototroch and giving rise to the pre-segmental adult prostomium;
(2) the soma, consisting of the prototroch, the mouth segment, and a growth zone which subsequently buds off trunk segments homologous with the mouth segment;
(3) the post-segmental pygidium, lying behind the growth zone.

It has thus been generally accepted that the prostomium is a definable pre-gmental unit comparable in all polychaetes and that the mouth segment is he first segment of the trunk.

Woltereck (1905) offered a different interpretation of the composition of the ochophore. He divided the larva into two adult rudiments separated by a one of larval tissue always represented at least by the prototroch:

(1) the anterior rudiment, giving rise to the prostomium (*Kopfzapfen*) containing the brain;
(2) the posterior rudiment, representing the trunk, and in later develop-

ment forming: (*a*) the mouth-region (*Schlund-region*), giving rise to th peristomium, sometimes in combination with one or more of the se ments laid down behind it; (*b*) the trunk, consisting of a number typical segments; and (*c*) the unsegmented pygidium, lying behind th segment-forming growth zone.

Woltereck, however, once more considers the prostomium to be a definab presegmental unit and further implies in the arguments supporting his thes that the mouth region, while distinct from the trunk, again represents a mod fied single segment. His interpretation of the pygidium as a part of the trun ignores its importance as the post-segmental region derived from an unseg mented ancestor.

The results obtained for *S. armiger* throw doubt on the validity of both th classical and Woltereck's interpretation of the trochophore. In this species ar in all polychaetes which have been examined in sufficient detail, the fir definitive segment is formed only when the growth zone becomes active ar the larva starts to elongate. Prior to this the trochophore can be divided int

(1) the prototrochal region, which subsequently forms the prostomium ar mouth region;
(2) the growth zone, from which all the trunk segments arise;
(3) the pygidium, the post-segmental region of the body lying behind th growth zone.

The further development of each of these parts of the body is discusse below.

The development of the trunk in polychaetes

The ectoderm of the trunk segments. In all polychaetes the ectoderm of th trunk segments arises from a prepygidial growth zone which in *Podark obscura* (Treadwell, 1901), *Neanthes succinea* (= *Nereis limbata*) (E. B. Wilso 1892), *Arenicola cristata* (Child, 1900), and *Amphitrite ornata* (Mead, 1897 as in *S. armiger* (p. 106), has been shown to originate from 2d. Such an origi of trunk ectoderm is probably the rule for polychaetes, although there ma be some variation in species with microlecithal eggs and a small 2d cell. I *Polygordius* (Woltereck, 1904), the trunk ectoderm is formed from descendan of 2d, 3c, and 3d. Eisig (1898) described a 2d origin of ectoderm in *Capitell capitata*, but also assigns a similar role to 4d, probably erroneously.

Previous investigations have shown that the trunk ectoderm in polychaet gives rise to the epithelium of the trunk segments, the chaetal sacs, and th ganglia of the ventral nerve-cord. This has been confirmed in *S. armig* (pp. 129–32). Typically, as in the latter species, the chaetal sacs and segment ganglia develop evenly from before backwards. In nereids and serpulids tho of the first three trunk segments develop precociously and simultaneously.

The mesoderm of the trunk segments. It has been demonstrated for a larg number of polychaetes that the trochophore possesses a pair of solid ventro lateral mesodermal bands budded off from pole cells or teloblasts situated i

he region of the prepygidial growth zone. In 9 species, including *S. armiger* p. 108), the origin of the pole cells has been traced to the blastomere 4d.

The mesodermal bands continue to elongate as the trunk segments begin o form, and give rise in each segment to a pair of hollow mesodermal somites compare p. 108). Little evidence is available, however, as to the origin of the nore posterior parts of the mesodermal bands. In *S. armiger* (pp. 108, 139), s in *Arenicola cristata* (Lillie, 1905), they are formed entirely from descen- ants of 4d, although the pole cells themselves soon lose their identity in iving rise to a mass of actively dividing prepygidial mesoderm cells. In *'apitella capitata* (Eisig, 1898) the somites of the 13 segments formed before hetamorphosis are budded off by the pole cells, but Eisig could not subse- uently distinguish mesoderm from ectoderm in the growth zone. In *Owenia usiformis* (D. P. Wilson, 1932) the teloblasts give rise to the first two pairs of runk somite rudiments before dividing each into two cells from which the hesoderm of the remaining pre-metamorphic segments is formed. The origin f the mesoderm in segments formed after metamorphosis was not traced. In *Amphitrite ornata* (Mead, 1897) the 4d mesodermal pole cells lose their dentity in a mass of undifferentiated mesoderm at the posterior ends of the aesodermal bands during formation of the first 5 segments, but the subsequent ourse of segment formation is not known. The mesodermal bands in *Lopa- orhynchus* (Meyer, 1901) are budded off from two groups of undifferentiated ells which migrate inwards from the trochophore ectoderm and which Meyer himself interpreted as descendants of pole cells, although Iwanoff 928) claimed that this condition represents an ectodermal origin of segmental aesoderm.

For serpulids, Iwanoff (1928) showed that the mesoderm of the three egments laid down before metamorphosis in *Eupomatus* (= *Hydroides*) *ncinatus* is formed by the mesodermal teloblasts; but that of the post- etamorphic segments arises by immigration of cells from the dorsal region f the ectodermal growth zone. In *Protula elegans* (= *Psygmobranchus pro- nsus*) (Meyer, 1888), the teloblasts give rise to the mesoderm of 5 segments rmed before metamorphosis. The mesoderm of the more posterior segments budded off, according to Meyer, from residual mesoderm at the posterior nds of the mesodermal bands, an apparent contradiction of Iwanoff's results. vidence presented by Meyer in support of his claim cannot, however, be ccepted without question.

For Nereidae no reliable evidence exists on the origin of the segment esoderm. The work of Salensky (1882) on *Perinereis* (= *Nereis*) *cultrifera* nd of E. B. Wilson (1892) on *Neanthes succinea* (= *Nereis limbata*) indicates at the 4d mesodermal teloblasts give rise at least to the somites of the first ree segments, but the origin of the mesoderm of the further segments rmed after metamorphosis has never been described.

Two further claims to an ectodermal origin of somite mesoderm have been ade. Häcker (1895) so described the formation of the mesoderm of the eighth nd succeeding (post-metamorphic) segments in *Polynöe*, but since his figures

show that no cell boundaries could be seen in his preparations, this claim unacceptable without further proof. He made no comment on the origin the mesoderm of the first 7 segments. Iwanoff (1928), in a species of *Spi* described the mesoderm arising from a distinct group of cells of unknow origin at the 5–6 segment stage, but with 20 to 25 segments present, cells we migrating inwards from the ectodermal growth zone to form the somite Once again the evidence cannot be accepted without re-investigation, sinc Iwanoff's figures are unconvincing.

The origin of the somite mesoderm has not been investigated in othe polychaetes. For *Polygordius*, Woltereck (1905) showed that all the somite formed before metamorphosis arise from the 4d teloblasts. Further develop ment of the segments after this time was not studied. The general conclusio to be drawn is that the somites of segments formed before metamorphosis i polychaetes arise from the 4d mesodermal teloblasts or a group of the descendants in all species. The somites of the later segments originate fro the same source in *S. armiger* and *Arenicola cristata*. In *Eupomatus uncinatu* they arise from the ectodermal growth zone by immigration. In other specie evidence on their origin is either insufficient or wanting.

Mesodermal segmentation precedes ectodermal segmentation in *S. armige* (p. 108) and in all other known forms except the Nereidae and Serpulida In these families the 4d mesodermal bands have been shown by vario authors to remain unsegmented during the precocious differentiation c ectodermal chaetal sacs and ventral ganglia in the first three (sometimes fou trunk segments. Only when this differentiation is well advanced do the divide simultaneously into three (or four) corresponding pairs of somite Here, therefore, the determination of segmentation is apparently ectoderma Whether the mesoderm of the more posterior segments is delineated befo the ectoderm in nereids or serpulids is not clear.

In polychaete species with a large blastocoel extending into the growi trunk, e.g. *Owenia fusiformis* (D. P. Wilson, 1932), *Eupomatus* (= *Hydroide uncinatus* (Iwanoff, 1928), *Pomatoceros triqueter* (Segrove, 1941), the paire somites of the anterior trunk segments are formed initially as solid blocks mesoderm cut off from the mesodermal bands. Then, as they develop coelomi cavities, they expand and obliterate the blastocoel. Where, however, develop ment from a yolky egg leads to early disappearance of the blastocoel in compact embryo, as in *S. armiger* (p. 108), *Arenicola cristata* (Lillie, 1905 and other species, the paired cavities of the segmental somites appear first i the unsegmented mesodermal bands, and the walls of the distinct somite become organized round them. An indentical condition has been demon strated in *Peripatopsis* (Manton, 1949) and *Estheria* (Cannon, 1924), both wit developmental patterns influenced by yolk.

Primary and secondary segmentation. Investigation of the pattern of segmen formation in *S. armiger*, in particular the mode of origin and developmen of the paired mesodermal somites and their relationship to the ectoderm allows the opportunity for a critical reappraisal of a controversial topic i

olychaete embryology, Iwanoff's theory of primary and secondary segmentation (Iwanoff, 1928). It has been clearly demonstrated in recent years, especially by Manton (1949), that this theory cannot now be upheld for arthropods; nd its application to polychaetes, one of the mainstays of Iwanoff's argument, as been similarly placed in doubt. Iwanoff based his argument on two main nes of evidence: (*a*) the frequent and undoubted occurrence of heteronomy the formation of body segments in two successive series, primary and secondary, differing in development and often in origin) among the segmented nvertebrates; (*b*) the pattern of heteronomy in the serpulid *Eupomatus ncinatus*, which he himself investigated. Iwanoff's results for this species ave already been outlined above. His interpretation of them may be summarized as follows:

(1) The serpulid three-segment larva is the primitive larva of polychaetes. t has three primary segments, in which segmentation arises simultaneously n the ectoderm, being secondarily superimposed on the mesoderm of the 4d nesodermal bands.

(2) Secondary segments are added to the body from a prepygidial growth one, their mesoderm being laid down in segmental units from the start. Primitively such mesoderm arises from 4d, but secondarily, as in *Eupomatus*, ssociated with the functional differentiation of all the 4d mesoderm in the primary larval body, it may be formed by ectodermal immigration from the prepygidial growth zone.

Thus according to Iwanoff the polychaete trunk is made up of segments of wo distinct kinds. In a later paper (1944) he declared such heteronomy to be new principle of morphology, applicable to all segmented animals.

Manton (1949), on the other hand, on the basis of investigation into the mbryology of Onychophora and arthropods, was led to the conclusion that he primitive mode of segment formation in invertebrates is probably evenly rom before backwards, as in *Peripatus*. Here the segments all develop in the ame manner, with mesoderm arising from a blastoporal area (corresponding o the polychaete 4d) and segmenting before the ectoderm. Where heteronomy s found in arthropods, it is as a secondary phenomenon associated with pecialization of the most anterior trunk segments for active larval life, e.g. n the crustacean nauplius. In the Crustacea heteronomy persists even when n increase in the amount of yolk in the egg renders it functionally unnecessary naupliar segments of Malacostraca, Manton, 1928, &c.), and as a corollary, he mesoderm of the secondary (post-naupliar) segments may arise from a ew source secondarily evolved (e.g. teloblasts of *Hemimysis* (Manton, 1928) nd other Malacostraca). If heteronomy were primitive, as Iwanoff supposed, ne would expect to find some trace of it in Onychophora, since the Crustacea how that once present it is not eliminated under the subsequent influence of olk.

Returning to polychaetes, it is undeniable that *Eupomatus* shows heteronomy. Its first three trunk segments are functionally differentiated as primary egments, in a manner assisting the planktotrophic efficiency of the larva,

before the more posterior segments begin to develop (compare the nauplius). Furthermore the rapid development of functional ectodermal segmental larval organs in these segments (chaetae which impart rigidity to the swimming larva and can be erected in defence, ciliated bands which provide the motive force in swimming) results in an apparent ectodermal determination of segmentation, the formation of corresponding somites in the unsegmented 4d mesodermal bands being somewhat delayed. Finally, the secondary segments have mesoderm arising from a different source (by ectodermal immigration) and segmenting before the ectoderm.

Eupomatus has a microlecithal egg, so that ability to feed at an early stage in development is a functional necessity. How does it then compare with other serpulids, in which the egg is yolky and the larva lecithotrophic? Salensky (1882), Meyer (1888), and Schiveley (1897) have shown that in such species the three specialized primary segments persist, though in a reduced form. This condition is analagous to that of Crustacea. If Iwanoff's assumption of the primitiveness of the specialized three-segment larva in polychaetes is true, therefore, it is to be expected that whatever the yolkiness of the egg, some trace of primary segments will be apparent in the development of all polychaetes. In their absence, Manton's argument for Onychophora becomes applicable to polychaetes, and this indicates that heteronomy in these, as in arthropods, is a secondary feature.

The main criterion for recognizing primary segments in polychaetes is simultaneous ectodermal determination of their segmentation. Iwanoff himself realized that a 4d origin of all segmental mesoderm is to be accepted as primitive for the group, and admitted that in the ectodermal origin of their mesoderm the secondary segments of *Eupomatus* are specialized. In *Scoloplos armiger*, however, the pattern of segment formation follows exactly that of Onychophora. Every trunk segment contains a pair of 4d mesodermal somites and, in addition, they all develop in strict succession, the mesoderm segmenting before the ectoderm. There is no trace of primary and secondary segmentation (heteronomy) in Iwanoff's sense. The only hiatus in segment formation in *Scoloplos* occurs during the transition from yolk feeding to active feeding, this being a common phenomenon in polychaetes and not an indication of a fundamental change in the mode of segment formation. For the one other polychaete in which the origin and development of the segments has been worked out in detail, *Arenicola cristata* (Lillie, 1905), the same conditions hold. Here again there is no trace of heteronomy. In fact the only polychaete family other than the Serpulidae to show heteronomy is the Nereidae, in which it has presumably evolved independently and where it once more persists even under the influence of yolk (Reish, 1954; Dales, 1950). It can be concluded therefore that heteronomy in polychaetes is not primitive, as Iwanoff suggests, but secondary, an adaptive response to planktotrophic needs, and that the primitive mode of segment formation is evenly from before backwards, the mesoderm segmenting before the ectoderm. Where heteronomy does occur, e.g. in Serpulidae and Nereidae, the mesoderm of the

econdary segments may have an origin different from that of the primary egments (e.g. *Eupomatus*) as it does in some Crustacea.

Other arguments can be adduced to support this conclusion, although the vidence is somewhat limited. Perhaps the most convincing examples are *Owenia fusiformis* (D. P. Wilson, 1932) and *Polygordius* (Woltereck, 1902, 904, 1905). Here the need for planktotrophic efficiency has been met, indeendently in the two species, in a different way, by elaboration of a highly eveloped trochophore with a large prototroch and capacious stomach. The pecializations involved do not touch the trunk segments and it is interesting o find that the first 11 trunk segments in *Owenia*, and the first 35 in *Polyordius*, develop in strict succession, the mesoderm arising from 4d and segenting before the ectoderm, e.g. in the primitive manner suggested by *Scoloplos*. There is no trace in either species of Iwanoff's three 'primary egments'. No doubt further detailed investigations of the development of ther species will confirm this conclusion for polychaetes in general.

Further development of the trunk mesoderm. It is accepted as characteristic or polychaetes that as the paired coelomic cavities of the trunk somites xpand, their walls differentiate into somatic, splanchnic, septal, and mesenerial mesoderm. When separate somite blocks are formed, the intersegmental epta arise by apposition of the anterior and posterior walls of adjacent blocks. When the paired coelomic cavities develop in unsegmented mesodermal ands, as in *S. armiger* (p. 108), the septa are formed in position between uccessive cavities. Similar considerations apply to the formation of mesenteries. The most anterior somites are usually laid down as lateral pairs which xtend dorsally and ventrally to meet in the mid-line above and below the gut, heir apposed walls forming the dorsal and ventral mesenteries of this region. More posteriorly, the somites usually arise from residual mesoderm already urrounding the gut, and the mesenteries are formed in position (e.g. *S. rmiger*, p. 109).

The relationship between the mesodermal somites and the adult 'middle ayer' organs of polychaete trunk segments has long remained controversial. There is general agreement that the walls of the somites form the splanchnic nd somatic peritoneum, the epithelia of the septa and mesenteries, and the walls of the blood-vessels (p. 153), and the results obtained for *S. armiger* upport this conclusion (pp. 133–9). The origin of dorsal and ventral longiudinal muscles from four rows of myoblasts in the somatic peritoneum has lso been described in every species so far examined. Bending of the trunk by he longitudinal muscles is one of the earliest muscular activities of the segnents in polychaetes, and in those species without larval musculature, such s *S. armiger*, it is the first observable contractile movement (p. 94). The levelopment of the longitudinal muscles appears to follow the same course n other species as in *S. armiger* (p. 137). They differentiate soon after somite ormation as thin fibrils closely applied to the ectoderm, several being formed n each myoblast. The myoblasts elongate and the fibrils fuse and run together with those of preceding and succeeding myoblasts to form multi-nuclear

muscle fibres. When the somites are differentiated in solid mesodermal bands as in *S. armiger* (p. 108), the rows of myoblasts remain unaffected by segmentation and the muscle fibres extend from the beginning across intersegmental boundaries. When separate somites are formed, the elongating fibres extend across these boundaries at a later stage of development. The distinct segmental activity of the longitudinal muscles in later life is evidently due to the arrangement of individual muscle fibres innervated by segmental nerves rather than to the obvious presence of such segmental muscle blocks as are found, for instance, in myriapods.

In *S. armiger* the somatopleure further gives rise to the circular muscles of the trunk (p. 138) and the musculature of the chaetal sacs (p. 138). Development of the circular muscles is very difficult to study in polychaetes and little evidence on their origin has been presented. According to D. P. Wilson (1936) the circular muscles in *Branchiomma vesiculosum* are formed, as in *Scoloplos*, in the somatopleure. Lillie (1905), however, claims an ectodermal origin of circular muscles in *Arenicola cristata*, although his figures suggest that this conclusion is unreliable. Meyer (1901) advocates an ectodermal origin of circular muscles in several species (see below).

Information concerning the origin of the chaetal sac muscles is more abundant. They have been shown to arise from somatic mesoderm in eight species including *S. armiger* (p. 138). Only Meyer (1901) denies this (see below).

The origin of the oblique, transverse, and dorso-ventral trunk muscles and the septal and mesenterial muscles of polychaetes has been little studied. In *S. armiger* the oblique, transverse, and dorso-ventral muscles arise from the somatic mesoderm (p. 138). For other polychaetes no reliable evidence is available on this point, although the mesodermal nature of these muscles has generally been assumed by workers on adult morphology. The septal muscles of *S. armiger* differentiate from cells of the septum itself. No muscles are formed in the mesenteries. Meyer (1901) has described the formation of septal and mesenterial muscles in other forms, arriving at a different conclusion as to their origin (see below).

The gut in polychaetes characteristically undergoes peristalsis along at least part of its length, but the origin of the muscles which cause this activity is virtually unknown. In *S. armiger* (p. 133) it has proved impossible to trace the development of the gut musculature. D. P. Wilson (1932) on *Owenia fusiformis* reports the formation of circular muscle-fibres in the splanchnopleure, but here they serve only to move blood in the sinus surrounding the gut. Meyer (1901) describes the origin of the gut musculature in *Lopadorhynchus*, *Protula*, and *Polygordius*, but from a source other than the splanchnic mesoderm (see below).

The suggestion that a large part of the polychaete trunk musculature originates not from mesodermal band-cells but from mesenchyme cells of an ectomesodermal nature was put forward by Meyer (1901) on the basis of his own investigations into the development of *Lopadorhynchus*, *Protula*

= *Psygmobranchus*), and *Polygordius*. Until these species have been re-xamined, however, the validity of this conclusion must remain in doubt. ctodermal trunk musculature is a well-known feature in certain Crustacea Cannon, 1926; Manton, 1928, 1934), but has never been seen in other olychaetes and certainly does not occur in *S. armiger*.

The development of the blood vascular system in polychaetes was reviewed y Hanson (1949), who supported Lang's theory (1904) that the haemocoel ccupies the site of the blastocoel and the walls of the blood-vessels are rmed by the basement membrane and cells of the mesodermal peritoneum. his conclusion is substantiated by the results obtained for *S. armiger* pp. 134–5).

In many polychaetes a large blood sinus is formed around the gut. In *tenodrilus branchiata* (Sokolov, 1911), *Arenicola cristata* (Lillie, 1905), *wenia fusiformis* (D. P. Wilson, 1932), *Protula elegans* (= *Psygmobranchus rotensus*) (Salensky, 1882), and *Salmacina dysteri* (Giard, 1876), and also in *olygordius* (Hatschek, 1878), this sinus is formed by separation of the planchnic mesoderm from the endoderm. In *Scoloplos armiger* a complete ut sinus is not developed, but in the dorsal mid-line the splanchnopleure eparates from the endoderm and the lateral walls of the resulting sinus come gether again above the gut to form the dorsal longitudinal blood-vessel ig. 18). The dorsal and ventral longitudinal vessels of *Polymnia nebulosa* = *Terebella meckelii*) (Salensky, 1883) and *Magelona papillicornis* (Buchanan, 895) and the ventral vessel of *Arenicola cristata* (Lillie, 1905) develop in the me way. The ventral vessel of *S. armiger* is formed by separation of the two mellae of the mesodermal ventral mesentery, as are the dorsal and ventral essels of *Ctenodrilus branchiata* (Sokolov, 1911), the dorsal vessel of *Arenicola ristata* (Lillie, 1905), and the ventral vessel of *Pomatoceros triqueter* (Segrove, 941). Since the gut sinus is considered to be a primitive feature in poly-naetes, it is possible that the dorsal and ventral vessels have evolved by nclosure of part of this sinus in the dorsal and ventral mid-lines. The gener-lly accepted view that the dorsal blood-vessel in polychaetes arises, like the rthropod heart, as a space left between the dorsal ends of the upgrowing egmental somites, finds no support in polychaete embryology. The arthropod ondition remains derivable from the polychaete condition, however, and the omology of the two organs is undisputed.

In *Arenicola cristata* (Lillie, 1905) and in *Polygordius* (Meyer, 1901) as well s in *S. armiger*, the segmental commissural vessels are formed by a partial eparation of the two epithelia of each intersegmental septum. Of the smaller essels, formed in *S. armiger* by separation of both splanchnic and somatic esoderm from endoderm, ectoderm, or adjacent mesoderm, no information available for other species except that in *Polygordius* the walls of the blood-essels in the blastocoelic pre-oral cavity arise from mesenchyme of unknown rigin (Woltereck, 1905).

The Development of the gut in polychaetes

In polychaetes the gut is formed from an ectodermal stomodaeum and a endodermal mid- and hind-gut rudiment. The mouth arises from the anterio end of the blastopore, which never closes completely (Delsman, 1916). Th anus develops at the point of fusion of the hind-gut and pygidial ectoderm with little or no proctodaeal ingrowth. In the primitive condition it is forme at the site of the posterior end of the blastopore, which either never close completely (*Podarke obscura*, Treadwell, 1901) or re-opens very soon afte blastoporal closure (*Eupomatus uncinatus*, Shearer, 1911; *Polygordius*, Wol tereck, 1904). When the presence of yolk delays the differentiation of the gu the anus opens late, as in *S. armiger*, but here again its site is approximatel that of the posterior end of the blastopore. In blastoporal closure the pre pygidial growth zone is laid down anterior to this point, and the anus alway lies behind the growth zone.

The stomodaeum. The stomodaeum has been shown to arise from cells of th second (other than 2d) and third quartettes in *Podarke obscura* (Treadwel 1901), *Neanthes succinea* (= *Nereis limbata*) (E. B. Wilson, 1892), *Capitell capitata* (Eisig, 1898), and *Polygordius* (Woltereck, 1904), as well as i *S. armiger* (Delsman, 1916). In *Arenicola cristata* (Child, 1900) it is formed b third quartette cells only.

Primitively, as in the serpulid trochophore, the stomodaeum differentiate rapidly into the functional larval pharynx, and in species in which a comple feeding trochophore is developed it retains this condition for a long tim (*Owenia fusiformis*, D. P. Wilson, 1932; *Polygordius*, Woltereck, 1904, 1905; When early development is influenced by the presence of yolk, as in *S armiger*, the stomodaeum forms initially as a solid ingrowth with a virtua lumen and becomes functional only later in development, when the yolk ha been resorbed (pp. 116–18).

The exact contribution which the stomodaeum makes to the adult gut ha been little studied. In *S. armiger* it forms the pharynx and the anterior end c the oesophagus. A very similar pharynx and oesophagus have been describe in *Aricia foetida* (Salensky, 1883; Schaxel, 1912) and *Haploscoloplos bustor* (Horn and Bookhout, 1950), but none of these authors distinguished ectc dermal from endodermal constituents in the differentiated gut. In *Platy nereis massiliensis* (= *Nereis dumerilii*) (Schneider, 1913) and in *Capitell capitata* (Eisig, 1898) the stomodaeum forms the pharynx and the oesophagu In the above species, in which feeding does not begin until the form and habi approximate to those of the adult, the cells of the stomodaeum differentiat directly into the epithelium of the adult fore-gut. In *Lopadorhynchus* (Kleiner berg, 1886; Meyer, 1901), *Polynöe*, *Nephthys*, and *Lepidonotus sp.* (Häcke 1896), *Spirorbis militaris* (= *Pileolaria militaris*) (Salensky, 1883), and *Poly gordius* (Meyer, 1901; Woltereck, 1905), the larval oesophagus develops a pa of evaginations, the cells of which replace those of the larval organ at meta morphosis. It is probably significant that these 'imaginal disks' are forme

nly in species in which active feeding begins very early and the stomodaeum functional in the trochophore.

The endoderm. It is well known that the endoderm in polychaetes is derived rom blastomeres 3A, 3B, 3C, and 4D, with contributions in some species rom 4d. In the typical planktotrophic trochophore it differentiates rapidly nto the functional stomach and intestine. As the trunk segments begin to rm the intestine lengthens and at metamorphosis the larval gut transforms irectly into the adult mid- and hind-gut (*Pomatoceros triqueter*, Segrove, 941; *Owenia fusiformis*, D. P. Wilson, 1932; *Polygordius endolarva*, Wol- reck, 1902). When the endoderm cells contain a proportion of yolk, a long ndodermal tube is formed by division of the cells, and differentiation of this ibe into oesophagus, stomach, intestine, and rectum then takes place . *armiger*, this account pp. 132–3; *Aricia foetida*, Schaxel, 1912; *Haplo- oloplos bustoris*, Horn and Bookhout, 1950; *Arenicola cristata*, Lillie, 1905; *xiothella mucosa*, Bookhout and Horn, 1949; and *Clymenella torquata*, ewell, 1951).

In *Capitella capitata* (Eisig, 1898) and *Platynereis massiliensis* (= *Nereis umerilii*) (Schneider, 1913), where the four primary endoderm cells contain n intense concentration of yolk, vitellophags appear and the gut develops in manner clearly analogous to that of yolky arthropods. In the *Polygordius olarva* (Woltereck, 1905), on the other hand, the endoderm gives rise to a ry large functional larval stomach, and an intestine growing with the trunk. hen metamorphosis occurs and the blastocoel is reduced, the larval stomach nnot be accommodated in the anterior end of the trunk. Its walls histolyse nd disappear and the adult gut is formed by union of the stomodaeal sophagus and the intestine.

As the trunk segments are successively formed the gut increases in length nd its different regions migrate backwards relative to the segments. In some olychaete species the adult position is quickly attained (e.g. *Clymenella rquata*, Newell, 1951), but in others, such as *S. armiger* (p. 141) the gut ntinues to change throughout life. The exact mode of migration remains bscure. Lillie (1905) on *Arenicola cristata* and Bookhout and Horn (1949) on *xiothella mucosa* ascribed it to a redifferentiation of cells at the anterior end each region into cells of the region in front of them (e.g. stomach into sophagus). D. P. Wilson (1932, 1936) on *Owenia fusiformis* and *Branchiomma siculosum*, on the other hand, suggested that the gut slides freely within the lanchnopleure as it grows. For *S. armiger* no definite conclusion has been ached on this point (p. 141), but it is not unlikely that both rediffer entiation nd sliding are involved.

The proboscis. Although some form of proboscis is found in many poly- naetes, the manner of its development has been very little studied. In . *armiger* a pre-adult and an adult proboscis are developed successively . 142). The adult proboscis, unique to ariciids, develops in *S. armiger* and *A. foetida* (Eisig, 1914) as a series of ciliated oesophageal evaginations hich can be everted through the mouth. The pre-adult proboscis, later

completely resorbed, is functionally similar to the adult proboscis in othe polychaete families such as the Cirratulidae, Terebellidae, and Oweniidae forming a protrusible lower lip, but little uniformity of structure or develop ment can be discerned in the different families.

In *S. armiger* the pre-adult proboscis apparatus comprises the proboscis skeleton lying ventro-posteriorly against the stomodaeum, proboscis retractor muscles, and a distinct non-coelomic proboscis cavity (pp. 121–3). This part of the apparatus originates from two cell masses descended from cells of the second and third quartettes, and lying initially on each side of the stomodaeum (p. 116). With it are associated various muscles (p. 128) differentiated from cells of the mesodermal bands, descended from 4d, and two diverticula of the posterior wall of the stomodaeum. A similar pre-adult proboscis is found in *Aricia foetida* (Eisig, 1914) and *Haploscoloplos bustoris* (Horn and Bookhout 1950) but its development has not been described. In the cirratulid *Ctenodrilus branchiata* (Sokolov, 1911) the same type of proboscis again occurs with skeletal plate behind the stomodaeum and an associate set of retractor muscles. According to Sokolov the whole apparatus is formed from mesoderm. In *Cirratulus cirratus* (Stephenson, 1950), the proboscis assists in locomotion in the early stages of development, as in *S. armiger* (p. 100).

The only detailed study of proboscis development in polychaetes, other than the present work, is that of D. P. Wilson (1932) on *Owenia fusiformis*. This species shows many differences from *S. armiger* but also certain interesting resemblances. The proboscis skeleton (the eversible part of the 'buccal organ') is formed in *Owenia* as a thickened ectodermal invagination which comes to lie in the postero-ventral wall of the oesophagus, just inside the mouth. With it is associated a complex musculature derived partly from the mesodermal somites of the cephalized first trunk segment and partly from two cell masses of unknown origin (but almost certainly not 4d mesoderm) lying initially on each side of the oesophagus (compare *Scoloplos armiger*). In the just-metamorphosed adult the buccal organ muscles are enclosed in a separate cavity, the buccal coelom, formed by confluence of the coelomic cavities of the first trunk somites with a pair of cavities formed in the oesophageal cell masses (compare *S. armiger*). The buccal coelom later becomes part of the adult head coelom (p. 161).

Thus in two species at least a pair of cell masses on each side of the stomodaeum, descended definitely in one and probably in the other from cells other than 4d, takes part in formation of the proboscis apparatus, including its musculature.

The polychaete pygidium

A post-segmental pygidium, lying behind the segment-forming growth zone and bearing the anus, is characteristic of polychaetes. It arises from ectodermal cells laid down during blastoporal closure, and in *Podarke obscura* (Treadwell, 1901), *Neanthes succinea* (= *Nereis limbata*) (E. B. Wilson, 1892), *Arenicola cristata* (Child, 1900), *Clymenella torquata* and *Amphitrite ornata*

Mead, 1897), as well as in *S. armiger* and in *Polygordius* (Woltereck, 1904), s cells have been shown to take origin from 2d. The only mesoderm so far iscovered in the pygidium is a part of the mesoderm of the growth zone. here does not appear to be any specific pygidial mesoderm in polychaetes. The telotroch is also essentially a pygidial structure. While it persists, it eparates the cells of the adult pygidium from the ectoderm of the growth one. In *S. armiger* and in *Amphitrite ornata* (Mead, 1897) the telotroch has een shown to consist of four ciliated cells, two dorso-lateral and two ventro-teral, descended from 2d, resorbed at metamorphosis.

he polychaete head

The head in polychaetes is generally taken to include the prostomium and eristomium, which for the adult were defined by Dales (1952) as follows:

(1) prostomium: that region which lies anterior to the mouth and contains the brain, bears the eyes and various appendages, and shows no obvious signs of a segmental nature. Its composition is disputed, but whatever the structure of the prostomium may be, it is a comparable unit throughout the polychaetes.

(2) peristomium: a variable structure which should always be defined for individual species. It often includes more than one segment, but typically is the first segment of the body, surrounding the mouth.

The present study of *S. armiger* has produced results which enable this efinition to be re-examined from an embryological standpoint.

It may be taken as certain that in all polychaetes the prototrochal region . 146) becomes externally divided into an anterior prostomium, containing ie brain, and a mouth region which lies in front of the first trunk segment. he only parts of the central nervous system in the mouth region are the aired circumpharyngeal commissures connecting the brain with the ventral erve-cord. The mouth region may give rise directly to the adult peristomium, ut in some species it fuses with the first trunk segment to form the latter. he first ganglion of the ventral nerve-cord (the sub-oesophageal) may then e in the peristomium, as in *Capitella capitata* (Eisig, 1898). Here the chaetal acs of the first trunk segment persist and the peristomium is chaetigerous. n nereids also the chaetigerous first trunk segment fuses with the mouth egion (various authors), but its chaetal sacs are resorbed and the peristomium achaetous. Once again, however, it contains the sub-oesophageal ganglion. n serpulids, Iwanoff (1928) for *Eupomatus* (= *Hydroides*) *uncinatus* and Meyer (1888) for *Protula elegans* (= *Psygmobranchus protensus*) describe the haetigerous first trunk segment losing its chaetal sacs and becoming the collar egion of the head; although Segrove (1941) reports for *Pomatoceros triqueter* that ie collar arises in front of the first trunk segment, i.e. on the mouth region. n *Owenia fusiformis* (Oweniidae, D. P. Wilson, 1932) the achaetous first unk segment early forms part of the peristomium, as it does in *Polygordius* Woltereck, 1905) and the peristomium contains the sub-oesophageal ganglion.

In *S. armiger* the head develops in a different way. The changes which undergoes are summarized in fig. 21. The achaetous first trunk segment (th metatrochal segment) fuses with the mouth region to form the adult per stomium, but at the same time its ganglion fuses with that of the second trun segment (chaetiger 1) to give the sub-oesophageal ganglion in chaetiger 1.

Thus an examination of the anterior end in adult polychaetes cannot provid reliable information on the composition of the head in segmental terms. Th question therefore remains how the prostomium and the mouth region, whic

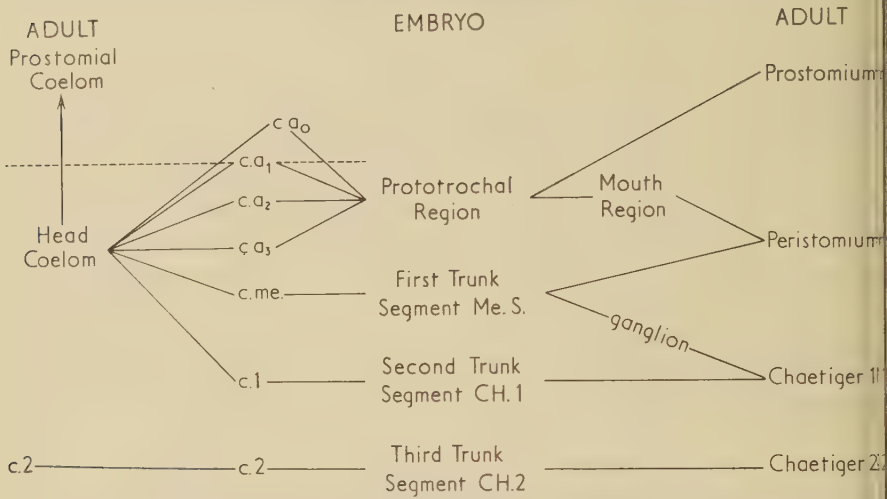

FIG. 21. Development of the head in *S. armiger* (schematic).
c 1, *c 2*, coelomic cavities of chaetigers 1, 2; ca_0, anterior median coelomic cavity; ca_1, ca_2, ca_3 paired lateral coelomic cavities of prototrochal region; *cme*, coelomic cavity of metatrocha segment.

form the original anterior end in development, are to be interpreted in term of a distinction between segmental and pre-segmental units of the bod (pp. 145–6). Manton (1949) points out that such a question can only b answered in embryological terms, but the available evidence on the develop ment of the prototrochal region is limited. The problem is further complicate by the fact that the prototrochal region develops initially as the body of th trochophore and therefore includes larval as well as adult tissue.

The work of various authors has shown, and the present study confirm (pp. 111–16), that the ectoderm of the prototrochal region develops in tw parts. Anteriorly it is formed by the first quartette of micromeres, of which the apical daughter cells $1a^1$ to $1d^1$ form the apical plate while the vegeta cells $1a^2$ to $1d^2$ form the primary prototroch cells. Behind the primary proto troch the ectoderm arises from those descendants of 2d, anterior to th ectoteloblast ring, which cover the embryo during blastoporal closure Descendants of $1d^1$ migrating posteriorly through the dorsal gap in th prototroch may also be added to them (Treadwell, 1901).

Primitively the apical plate gives rise to the cerebral ganglion rudiment and the differentiated ectoderm of the larval episphere. The development of the cerebral ganglion from apical plate ectoderm, independently of the ventral nerve cord, has been recorded for all species examined. The same ectoderm also gives rise to the epithelium of the adult prostomium, from which the brain rudiment gradually separates in the same manner as the ventral ganglia of the segments separate from the segmental ectoderm (p. 115). In trochophores which approximate to the ancestral form, such as those of *Pomatoceros triqueter* (Segrove, 1941) and *Branchiomma vesiculosum* (D. P. Wilson, 1936), it is generally agreed that the larval episphere ectoderm is lost at metamorphosis and that the only products of the apical plate in the adult are the prostomial ectoderm and brain. In *Owenia fusiformis* (D. P. Wilson, 1932) and *Polygordius* (Woltereck, 1902, 1905), where the trochophore is relatively large, the prostomium arises from only a small part of the apical plate.

In species with large yolky eggs, on the other hand, e.g. *S. armiger* (this account), *Capitella capitata* (Eisig, 1898), little or no larval episphere ectoderm is developed and the whole of the apical plate gives rise to adult tissue. It has previously been accepted for several species of this kind that, as in the species with a differentiated trochophore, the prostomium is the sole product of the apical plate in the adult; but in *S. armiger* part of the apical plate also contributes to the ectoderm of the mouth region (p. 115; see below).

The brain becomes connected with the ventral nerve cord by a pair of circumpharyngeal commissures which grow out from its neuropile, traverse the mouth region on each side between the ectoderm and the somatic mesoderm, and run into the neuropile of the ganglion of the first trunk segment. In forms with a functional trochophore (e.g. *Pomatoceros*, *Owenia*, *Polygordius*) the circumpharyngeal commissures develop early. In yolky forms they do not appear until several segments have been formed (12 in *S. armiger*).

Thus the prostomium and cerebral ganglion of polychaetes must be considered as pre-segmental. They develop in front of the anterior ends of the mesodermal bands in which segmentation arises, and mesoderm only invades the prostomium secondarily (see below). Further, the cerebral ganglion has a similar origin in platyhelminths and molluscs, related but unsegmented animals. The pre-segmental nature of the polychaete prostomium has been assumed by embryologists for many years and also by morphologists such as Holmgren (1916) and Hanström (1928), who have concluded that the differentiation of the polychaete brain into paired ganglia is associated with the development of paired sense organs in this region. Other morphologists, however, have endeavoured to interpret the polychaete brain as either a single unit homologous with the ventral ganglia of the trunk segments or as a number of cephalized segmental ganglia. The latter interpretations involve unwarrantable assumptions and ignore all the embryological evidence given above. The theories of Ferris and Henry (Ferris, 1953; Henry, 1947) and that of Raw (1949) require, for instance, that the polychaete proboscis shall consist of one or more 'segments', supposedly inverted body segments. The present account

shows that the polychaete stomodaeum, from which the proboscis in questio develops, bears no relation to segmentation. Manton (1949) and Hedgepet (1954) have adduced many other reasons for rejecting these theories.

The significance of the mouth region in relation to segmentation is mo difficult to assess than that of the prostomium, owing to its complex develop ment and to lack of evidence. Primitively, as in serpulids, the ectoderm of th mouth region is formed from hyposphere ectoderm anterior to the presump tive segmental growth zone, while between this ectoderm and the prototroc there lies a region of differentiated larval ectoderm lost at metamorphosis. I *Owenia fusiformis* (D. P. Wilson, 1932) and in *Polygordius* (Woltereck, 190 1905) very little of the ectoderm of the hyposphere represents future adu tissue, and the larval hyposphere ectoderm is extensive and well develope Conversely, in *S. armiger* (pp. 115–16) and other forms with a large yolky egg all the hyposphere ectoderm anterior to the growth zone forms ectoderm o the mouth region, and only the prototroch disappears at metamorphosis. Th ectoderm of the mouth region is thus formed from ectoderm cells which cove the embryo during blastoporal closure, and whether all or part of these cell persist into the adult depends on the relative suppression of the trochopho as a feeding stage. It is important to notice that the first segmental ganglio of the ventral nerve-cord arises in the first ectoderm laid down by the seg mental growth zone, i.e. the ectoderm of the first trunk segment. At no stag does the rudiment of a ganglion exist in the ectoderm of the mouth region. would therefore seem that the ectoderm of the mouth region cannot be cor sidered as segmental ectoderm and that this region is not homologous wit the trunk segments in polychaetes. Further, in *S. armiger* the ectoderm of th anterior part of the mouth region is formed by cells of the apical plate posterio to the developing prostomium, and more detailed examination may reve a similar condition in other species. The implication of this is that the pro stomium is not the sole definable pre-segmental unit in polychaetes, but tha the mouth region can also be considered as pre-segmental. Such a conclusio is further borne out by the mode of development of the mesoderm in th mouth region.

The main criterion for interpreting the mouth region as the first segmen of the trunk has previously been that it contained a pair of hollow mesoderma somites (e.g. Goodrich, 1897). The evidence for such an interpretation wa assessed for polychaetes with a simple planktotrophic trochophore (e.g serpulids) by Meyer (1901). Although the results which he reviewed wer perhaps not wholly reliable, no more recent work has been done on thes forms and the extent of our present knowledge may be summarized as follow

When the trunk segments begin to develop, the prototrochal region anterio to them is occupied by a large blastocoel containing various mesenchyma elements and surrounding the anterior end of the gut. The mesodermal pol cells do not become active until just before the ectoderm of the first trun segment begins to form, so that the anterior ends of the mesodermal banc project only a short way into the hyposphere of the trochophore body and d

t reach as far as the mouth. When the mesodermal bands segment into ired somites, the anterior ends of the bands form a pair of somites in front those of the first trunk segment, i.e. lying in the blastocoel of the future outh region (fig. 22, A). This pair of somites becomes hollow and expands, owing forwards on each side of the pharynx as the larval tissues of the ochophore are lost at metamorphosis, to meet in front of the mouth, forming e coelom of the peristomium. Later the peristomial coelom extends forward neath the brain into the prostomium.

Meyer believed that the muscles of the body wall in the mouth region and ose of the prostomium arose from mesenchyme cells pressed between the oderm and the expanding coelomic peritoneum, while those of the pharynx re formed from cells of the stomodaeal pouches (p. 154), covered by anchnic peritoneum. He also described a similar development of the soderm of the mouth region in *Polygordius*. Woltereck (1905), however, ted that the pair of somites which were formed in this region in *Polydius* remained post-oral and that the large pre-oral cavity behind the ebral ganglion was blastocoelic in origin.

Since 1901, further evidence on the development of the mesoderm in the outh region in polychaetes has been provided only by D. P. Wilson (1932) *Owenia fusiformis* and by the present account for *S. armiger*.

In *Owenia*, where the mesodermal teloblasts become active very late in velopment, the most anterior of their products occupy the first trunk segnt and do not invade the mouth region. At the same time a large anterior dian cavity is formed in mesoderm of unknown origin (apparently ectosoderm) lying beneath the developing cerebral ganglion. The hollow nites of the first trunk segment combine with a pair of hollow mesodermal sses (again of unknown origin) lying on each side of the oesophagus to m the 'buccal coelom' (p. 156), and at metamorphosis this and the anterior dian cavity together form the coelom of the peristomium. In *Owenia*, refore, no segmental somites are formed in the prototrochal region g. 22, C).

Scoloplos armiger shows an opposite condition. In this species the mesomal teloblasts become active very early and the mesodermal bands grow ward on each side and meet in the mid-line in front of the mouth shortly r the first trunk segment begins to develop. The mesoderm so formed lies neath the ectoderm of the future mouth region (fig. 22, B). When the sodermal bands segment, it is found that three pairs of coelomic cavities formed in the prototrochal region in front of the first trunk segment g. 22, B), and also an anterior median cavity of which the walls are partly omesodermal (compare *Owenia*). A similar anterior median cavity was scribed in *Aricia foetida* and *Polymnia nebulosa* (= *Terebella meckelii*) by ensky (1883), while Eisig (1898) described four pairs of lateral coelomic ities in the prototrochal region in *Capitella capitata*. In *S. armiger* the ities later run together and become confluent with those of the metachal segment and chaetiger 1 to form the adult head coelom, which finally

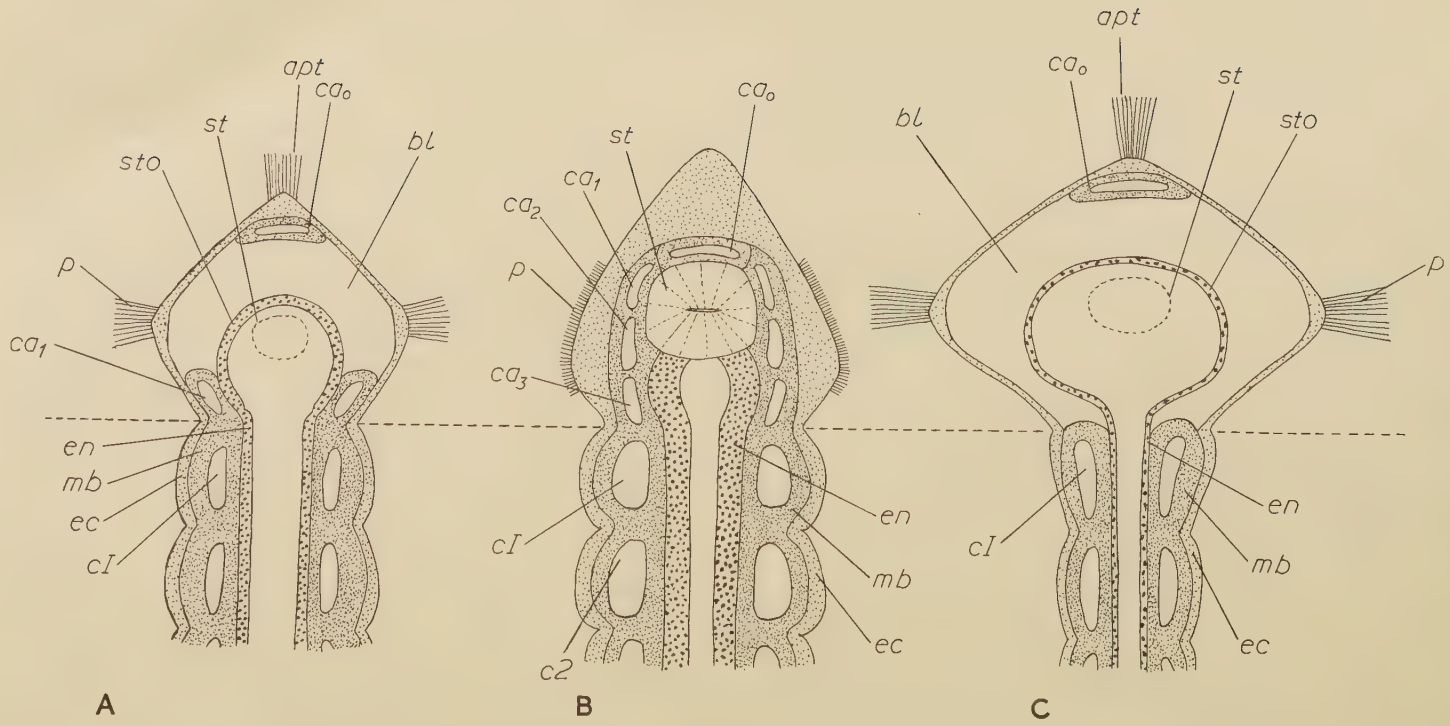

FIG. 22. Relationship between ectoderm and mesoderm in the prototrochal region in polychaetes (diagrammatic, not to scale). A, *Serpula*; B, *Scoloplos*; C, *Owenia*.

apt, apical tuft; *bl*, blastocoel; *c 1*, *c 2*, coelomic cavities of trunk segments 1, 2; ca_0, anterior median coelomic cavity; ca_1, ca_2, ca_3, paired lateral coelomic cavities of prototrochal region; *ec*, ectoderm; *en*, endoderm; *mb*, mesodermal band; *p*, prototroch; *st*, stomodaeum; *sto*, larval stomach.

vades the prostomium (fig. 21, p. 158). In contrast to the opinions of Meyer 901), the musculature of the prostomium and mouth region in *S. armiger* formed from cells of the mesodermal bands.

It has already been shown (p. 148) that segmentation in polychaetes is asically a mesodermal phenomenon, secondarily superimposed on the ecto- erm. This is borne out by the development of the mouth region, where the ctoderm is most probably pre-segmental and remains unaffected by the gmenting mesoderm (see above). The number of pairs of somites which vade the region depends on the condition of the trochophore larva. Primi- vely it appears to be one pair (fig. 22, A), although a more detailed study of rpulid development is required to establish this. When a very large blasto- el is developed in the trochophore, the 4d mesoderm may not invade the outh region at all (fig. 22, C), while in the lecithotrophic trochophore which ises from a large yolky egg, several pairs of somites may move forward into e mouth region (fig. 22, B) without inducing a corresponding segmentation the ectoderm. This cannot be thought of as a cephalization of segments such occurs in *Hanseniella* and *Pauropus* (Tiegs, 1940, 1947), since in these imals migration of ectoderm accompanies that of the mesoderm. As the olychaete themselves show, a cephalized segment, e.g. the first trunk seg- ent, which often becomes incorporated in the peristomium, can only be early defined if at some stage in its development it shows segmental features both mesoderm and ectoderm.

Thus the polychaete mouth region can be regarded as pre-segmental. Its velopment is complex, and in giving rise to the peristomium it may become sed with the first definable trunk segment. It is impossible, therefore, to nsider the polychaete peristomium as homologous either with the trunk gments which lie behind it, or with the antennal segment in Onychophora, suggested by Goodrich (1897). Manton (1949) showed that the latter seg- ent is undeniably homologous with its associate trunk segments, and upheld at the segmental composition of the head in Onychophora and Arthropoda n be given a unified interpretation along the lines suggested by Goodrich. polychaetes, however, the head has evolved its own specializations, and nnot be directly compared with the onychophoran-arthropod condition.

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